Binding of the hepatitis C virus E2 glycoprotein to CD81 and other receptors on immune system cells; and immunomodulatory functions of CD81 on innate cell subsets

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Abstract

Hepatitis C virus (HCV) persists in ~80 % of infected individuals. The mechanisms involved in HCV persistence and pathogenesis are not fully understood. The E2 envelope glycoprotein of HCV has been shown to bind to CD81, and it is hypothesised that E2 cross-linking of CD81 may have immunomodulatory effects. This thesis addressed the interaction of E2 with CD81 and other putative receptors on peripheral blood mononuclear cell (PBMC) subsets, and the potential for CD81 cross-linking to modulate innate responses.

Soluble truncated E2 glycoproteins were produced from several HCV genotype 1a and 1b strains, and their binding to PBMC subsets was investigated. CD81 was detected on all PBMC subsets, although expression levels on different cell types varied. By contrast, expression of SR-BI (another E2-binding protein) was restricted to monocytes and dendritic cells (DCs). H77c E2 showed a high level of binding to PBMCs, with binding to cell subtypes correlating with their level of CD81 expression. Other E2s showed a similar pattern of interaction with PBMC subtypes, but bound less well to all cell subsets.

The effect of antibody-mediated cross-linking of CD81 on NK cell and DC responses was investigated. No specific effect of CD81 ligation was seen on the response of NK cells to CD16 or cytokine stimulation. However, CD81 cross-linking specifically inhibited MICA-stimulated NK cell activation. Investigation of the effect of CD81 cross-linking on the response of monocyte-derived DCs to LPS, poly(I:C) and CD40 ligation did not reveal a role for CD81 in modulating DC responses to these stimuli, a

finding confirmed by examining the response of bone-marrow derived DCs from CD81 deficient mice to these stimuli.

If E2-CD81 interaction mediates immunomodulatory effects *in vivo*, the difference observed in the binding level of E2s from different HCV strains to PBMCs suggests that this may constitute one of the determinants of viral persistence/pathogenesis.

Publication

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Glossary of Terms

°C	degrees Celsius
µg	micrograms
µl	microlitre
aa	amino acid
Ab	antibody
AID	activation-induced cytidine deaminase
ADCC	antibody-dependent cellular cytotoxicity
ALT	alanine transferase
APC	allophycocyanin
APCs	antigen presented cells
APS	ammonium persulphate
BCR	B cell receptor
BMDCs	bone marrow-derived dendritic cells
bp	base pair
BVDV	bovine viral diarrhoea virus
CBA	cytometric bead array
CTL	cytotoxic T lymphocyte
DCs	dendritic cells
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3grabbing integrin
DC-SINR	liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulphoxide
EBV	Epstein-Barr virus
EIA	enzyme immunosorbent assay
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
FBS	foetal bovine serum
FCS	foetal calf serum

FITC	fluorescein isothiocyanate
GAGs	glycosaminoglycans
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage-colony stimulating factor
GST	glutathione S-transferase
HBV	hepatitis B virus
HCV	hepatitis C virus
HCC	hepatocellular carcinoma
HDL	high density lipoprotein
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
HSV	herpes simplex virus
HVR	hypervariable region
IFN	interferon
ICAM	intercellular adhesion molecule
IL	interleukin
IRES	internal ribosome entry site
IRF	interferon regulatory factor
ISDR	IFN sensitivity determining region
ITAMs	immunoreceptor tyrosine-based activation motifs
ITIMs	immunoreceptor tyrosine-based inhibitory motifs
KO	knock-out
KIRs	killer immunoglobulin-like receptors
LB	Luria-Bertani
LDL	low density lipoprotein
LEL	large extracellular loop
LPS	lipopolysaccharide
mAb	monoclonal antibody
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MICA/B	MHC class I-related chain A/B
MLV	murine leukemia virus

MW	molecular weight
NF-kB	nuclear factor-κB
NK	natural killer
NS	non-structural
NT	natural T
NTR	non-translated region
ORF	open reading flame
PAMPs	pathogen-asociated molecular patterns
PBMCs	peripheral blood mononuclear cells
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PePHD	PKR-eIF2α phosphorylation site homology domain
PHA	phytohemagglutinin
PRR	pattern-recognition receptor
PTKs	protein tyrosine kinases
RANTES	regulated on activation normal T-cell-expressed and secreted
RIG	retinoic acid-inducible gene
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEL	small extracellular loop
SR-BI	scavenger receptor class B type I
STAT	signal transducer and activator of transcription
TBK	TANK-binding kinase
TCR	T cell receptor
TEMED	N, N, N', N'-Tetramethylethylenediamine
TNF	tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TRIF	Toll-IL-1 receptor domain-containing adaptor-inducing IFN-β
ULBPs	UL-16-binding proteins
VLDL	very low density lipoprotein
VLPs	virus-like particles
VSV	vesicular stomatitis virus

Chapter 1 Introduction

1.1. Background

Hepatitis C virus (HCV) infection is a major public health problem. Approximately 170 million people worldwide are chronically infected with this virus (Cohen, 1999). HCV infection is characterised by an asymptomatic phase of acute infection followed by a high rate (70~80 %) of viral persistence, during which the development of chronic liver diseases such as hepatitis, cirrhosis and fatal hepatocellular carcinoma (HCC) is frequently observed (Alter, 1995; Marcellin, 1999). HCV infection is also associated with extra-hepatic diseases such as mixed cryoglobulinemia, membranoproliferative glomerulonephritis and B cell neoplasias (Ferri & Zignego, 2000). The high rate of persistence of HCV infection suggests that there are defects in the immune response mounted to the virus and/or the virus possesses effective immune evasion strategies.

HCV was first recognised as a distinct form of liver disease following the introduction of diagnostic tests for hepatitis A and B virus infections in the mid-1970s. The identification of HCV was a cutting-edge discovery, due to the use of direct cloning techniques without viral propagation in cell culture. The genome of HCV was first cloned in 1989 by screening a cDNA expression library derived from the plasma of a persistently infected chimpanzee that was inoculated with HCV patient serum, and this virus was identified as the major cause of blood transfusion associated non-A, non-B hepatitis (Choo *et al.*, 1989; Kuo *et al.*, 1989).

Although more than a decade has passed since the discovery of HCV, the only therapy available for this infection is treatment with interferon (IFN)-α with or without ribavirin. Although the introduction of pegylated IFN with ribavirin has improved the efficacy of treatment (Pearlman, 2004), it is often accompanied by side-effects and almost 50 % of infected patients do not normalise serum alanine aminotransferase (ALT) levels and fail to clear their infections. Currently, no vaccine is available to prevent HCV infection.

There is thus an urgent need to understand the molecular biology of HCV and the interaction between HCV and the host immune system in order to facilitate the development of vaccines and new treatments to combat this infection. However, studies of HCV virology and of viral persistence and associated pathogenesis are hampered by obstacles including the limited availability of *in vitro* cell culture systems supportive of viral replication and the lack of *in vivo* small animal models (chimpanzees are the only animals other than humans susceptible to HCV infection).

1.2. Virology of HCV

1.2.1. Genome

HCV is the only member of the genus *Hepacivirus* in the *Flaviviridae* family. This family includes two other genera, *Pestivirus* and *Flavivirus* (Rice, 1996; Robertson *et al.*, 1998). Other viruses belonging to the *Flaviviridae* family include the pestiviruses bovine viral diarrhoea virus (BVDV) and swine fever virus; and the flaviviruses Japanese encephalitis virus, West Nile virus, yellow fever virus and dengue virus.

The HCV particle is 40-50 nm in size and consists of an envelope, a nucleocapsid and a ~9.6 kb positive sense single-stranded RNA genome. The genome carries one open reading flame (ORF) flanked by non-translated regions (NTR) at the 5' and 3' termini. The transcript encodes a polyprotein of approximately 3000 amino acids (aa) that is co- and post-translationally cleaved into at least 10 structural and non-structural (NS) proteins by both host and viral proteases. The HCV structural proteins (core, E1, E2 and p7) are located at the amino (N) terminus, whilst the NS proteins (NS2, 3, 4A, 4B, 5A and 5B) are towards the carboxy (C) terminus (Fig. 1.1). In addition, a frameshift signal

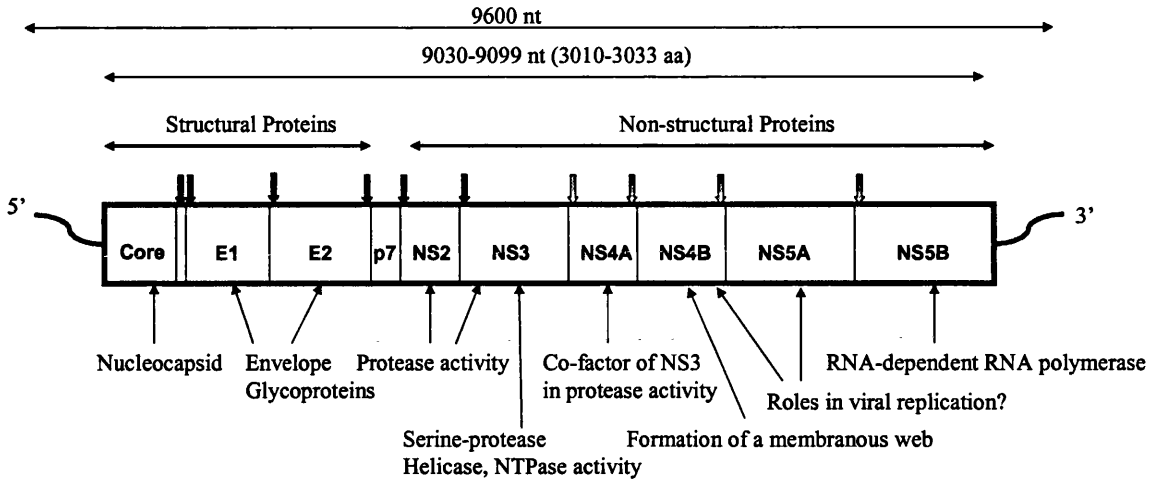


Figure 1.1. Genomic organisation of HCV.

HCV has a 9.6 kb positive stranded RNA genome encoding one polyprotein of about 3000 amino acids (aa). This polyprotein is cleaved into at least 10 structural and non-structural (NS) proteins by host and viral proteases. The four structural proteins are generated by cleavages mediated by host proteases. Endoplasmic reticulum signal peptidase cleaves the core/E1, E1/E2, E2/p7 and p7/NS2 junctions (red arrows) and the core protein is further cleaved by signal peptide peptidase (blue arrow). The core protein forms the viral nucleocapsid, and the E1 and E2 glycoproteins form heterodimers on the virion surface. NS2 forms a protease together with the N-terminal third of the NS3 protein and mediates the cleavage at the NS2/3 junction (purple arrow). The NS3 protein cleaves at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions (green arrows). Functions of the non-structural proteins are indicated on the diagram. (Figure adapted from Bartenschlager and Lohmann, (2000) and Reed and Rice, (2000))

during translation of the N terminal region of the polyprotein results in the production of the core protein and a small protein called frameshift (F) protein (Xu *et al.*, 2001).

1.2.2. Viral lifecycle

As cell culture systems allowing the propagation of HCV have only recently begun to be developed, HCV replication has mainly been studied using recombinant HCV proteins or deduced from existing knowledge about other viruses in the family *Flaviviridae* with similar genomic organisations and functionally homologous NS proteins (Bartenschlager & Lohmann, 2000). More recently, a highly efficient HCV replication model was developed based on the autonomous replication of engineered HCV subgenomic or genomic replicons in transfected Huh-7 cells (Blight *et al.*, 2000; Lohmann *et al.*, 1999). Although the development of replicon systems was of great value for understanding viral replication, none of the ongoing systems were able to produce viral particles or infectious virions. However, Heller *et al.* and Zhong *et al.* recently reported the development of a new HCV expression construct that supports not only high level RNA replication, but also the production and secretion of high titres of HCV virions (Heller *et al.*, 2005; Zhong *et al.*, 2005). Then Wakita *et al.* showed that secreted virus particles from *in vitro* culture were infectious for Huh-7 cells in an E2- and CD81-dependent manner and infectious for chimpanzee (Wakita *et al.*, 2005). This *in vitro* model of HCV production will be a valuable tool for the study of the HCV lifecycle, especially viral assembly and release, and secreted particles will be useful for the study of viral entry.

The first step in viral lifecycle is the attachment of the infectious particle to the host cell. HCV is an enveloped virus expressing two surface glycoproteins, E1 and E2 (described

later in detail). Viral attachment is thought to be mediated by binding of the E2 glycoprotein to host cell receptors, followed by a fusion event. The HCV envelope glycoproteins are thought to be class II fusion proteins (Yagnik *et al.*, 2000). Sequence analysis suggests that the E1 glycoprotein might contain a fusion peptide (Flint & McKeating, 1999). Conversely, structural analysis of fusion proteins from other viruses in the same family or other class II fusion proteins suggests that E2 may be the fusion protein (Yagnik *et al.*, 2000). It has been reported that HCV cell entry is pH-dependent (Bartosch *et al.*, 2003b; Hsu *et al.*, 2003), implying that the viral particles are internalised by receptor-mediated endocytosis and transported to acid-pH endosomal compartments where conformational change(s) occur in the glycoproteins and membrane fusion takes place.

Once inside the cytoplasm, the positive sense viral RNA genome is directly translated at the rough endoplasmic reticulum (ER). The 5' NTR contains an internal ribosome entry site (IRES) allowing ribosomes to bind in close proximity to the start codon of the ORF (Tsukiyama-Kohara *et al.*, 1992). The viral polyprotein is cleaved by host and viral proteases (Fig. 1.1). The cleavage of the structural proteins is mediated by host proteases. A signal peptidase present in the lumen of the ER cleaves at the core/E1, E1/E2, E2/p7 and p7/NS2 junctions (Cocquerel *et al.*, 2002). Production of the mature core protein results from further cleavage at the signal peptide by signal peptide peptidase located in the ER membrane (McLauchlan *et al.*, 2002). In addition, the core protein can be truncated by proteolysis at additional sites (Hussy *et al.*, 1996; Liu *et al.*, 1997) and some truncated forms of the core protein are translocated into the nucleus. The cleavage of the NS proteins requires two viral proteins: NS2 and NS3 (Whitney *et al.*, 2002). The NS2 protein participates in an autoprotease activity that is responsible

for cleavage between the NS2 and the NS3 proteins (Grakoui *et al.*, 1993). The NS3 protease cleaves at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions. The NS4A protein forms a stable complex with the NS3 protein to act as a co-factor for the NS3 protease activity. Most HCV proteins are integrated into the membrane of the ER due to the presence of transmembrane domains or ER retention signals (Dubuisson *et al.*, 2002).

A characteristic feature of the lifecycles of positive-strand RNA viruses is that they modify intracellular membranes to create a compartment in which RNA replication takes place. The compartment is called a replication complex. The HCV replication complex consists of cleaved HCV polyprotein products, particularly NS3-5B, the genomic template and altered cellular membranes. The formation of a replication complex allows tight contact between components of the replication machinery and the recruitment of membrane-associated host cell proteins that are necessary for viral replication, resulting in the efficient production of viral RNA. Recently, it was reported that HCV replication occurs on lipid rafts that may be recruited from the ER and/or the Golgi apparatus (Gao *et al.*, 2004; Shi *et al.*, 2003). The formation of the RNA replication complex on lipid rafts may be initiated by NS4B because it has the intrinsic property of anchoring to lipid raft membranes (Gao *et al.*, 2004) and induces specific modification of membranes to form a membranous web containing all of the structural and NS HCV proteins (Egger *et al.*, 2002). Since the HCV NS proteins are predominantly associated with membranes from the ER and/or the Golgi apparatus (Mottola *et al.*, 2002), it is likely that they are recruited to the replication complex on lipid rafts only when participating in RNA replication.

RNA replication involves several viral NS proteins: the NS5B protein acting as an RNA dependent RNA polymerase (Wang *et al.*, 2002); the NS3 protein having RNA helicase activity (Pang *et al.*, 2002); the NS4A protein augmenting the helicase activity of the NS3 protein (Pang *et al.*, 2002); and the NS5A protein, which is also involved in the synthesis of RNA (Hardy *et al.*, 2003). The synthesis of a full-length negative-stranded RNA template is a prerequisite for the synthesis of positive-stranded RNA transcripts and full-length progeny genomes.

It is assumed that the core protein plays a role in particle formation. The core protein has three distinct domains: a N-terminal domain that allows binding to ribosomes and RNA (Santolini *et al.*, 1994); a central domain that is responsible for the localisation of the core protein, which is predominantly in the cytoplasm where it is found attached to the ER membrane and lipid droplets (Hope & McLauchlan, 2000; McLauchlan, 2000; Moradpour *et al.*, 1996); and a C-terminal domain that contains the E1 signal peptide. Particle formation is initiated by the interaction of new viral RNA genomes with the core protein to form nucleocapsids. During virion assembly, the nucleocapsids are thought to associate with the cytoplasmic side of the ER membrane. It has been reported that the core protein interacts with E1 protein (Lo *et al.*, 1996), supporting the idea that the core protein is involved in virion assembly. Viral glycoproteins are retained at the ER, thus it is thought that the nucleocapsids acquire E1 and E2 glycoproteins by budding into the ER, and that viral particles are released via the host secretory pathway (Bartenschlager & Lohmann, 2000; Dubuisson *et al.*, 2002). In addition, although the role of attachment of the core protein to lipid droplets is not known, lipid droplets are thought to bud from the ER membrane (van Meer & Sprong, 2004), suggesting the possibility that they may provide a platform for the assembly of HCV particles.

Besides the proteins that form the particle, act as proteases or are involved in RNA replication, HCV expresses two additional proteins, the p7 and F proteins. The F protein, which is produced following a ribosomal frameshift during core protein translation, has been reported to be associated with the ER (Xu *et al.*, 2001), but the biological function of this protein is not known. The function of the small protein p7 is not fully understood either. It was recently suggested that p7 may function as an ion channel (Griffin *et al.*, 2003). Moreover, p7 appears to be necessary for particle infectivity in the chimpanzee model, and interacts with other genomic regions in a genotype-specific manner (Sakai *et al.*, 2003).

1.2.3. Genotypes and quasispecies

A distinctive feature of HCV is its genetic heterogeneity (Bukh *et al.*, 1995). Based on the sequences of the entire core and E1 genes or NS5B, the isolates around the world are classified into 6 major genetic groups (genotypes 1-6), and these genotypes are further divided into subtypes (a, b, c etc.) (Simmonds *et al.*, 1993). Each genotype differs at 30-35 % of nucleotide sites compared with <25 % divergence between subtypes (Simmonds *et al.*, 1993).

The global distribution of different genotypes shows distinctive geographical patterns (Fig. 1.2) (Forns & Bukh, 1999). Genotypes 1a and 1b are the most prevalent worldwide. Genotypes 1a, 1b, 2a, 2b, 2c and 3a account for the majority of infections in Europe, the U.S.A., Russia, Japan, China and Australia, where 1-2 % of the population is infected by HCV (Forns & Bukh, 1999; Pellicano *et al.*, 2004). Although genotype 3a has a world-wide distribution, subtypes of genotype 3 other than 3a are highly prevalent in South-East Asia and other parts of Asia such as India, Bangladesh and Nepal

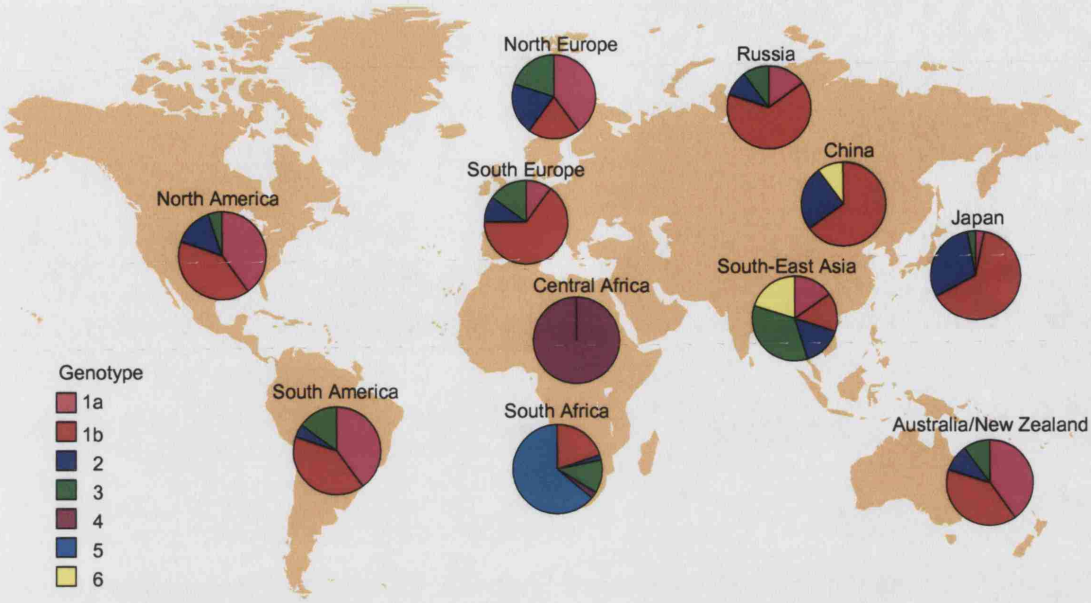


Figure 1.2. World distribution of HCV genotypes.

HCV can be classified into 6 genotypes. The distribution of HCV genotypes in different part of the world is shown. The distribution of the two most common subtypes, 1a and 1b, is also depicted. (Figure adapted from Forns and Bukh (1999))

(Farci & Purcell, 2000). Genotype 4a is mainly restricted to Egypt, where more than 24 % of the population is estimated to be infected by HCV (Bukh *et al.*, 1995; Cohen, 1999) and other subtypes of genotype 4 account for the majority of infections in the Middle East and central Africa (Chamberlain *et al.*, 1997b; Xu *et al.*, 1994). Genotype 5a is principally restricted to Southern Africa (Chamberlain *et al.*, 1997a) and genotype 6 to South-East Asia including Hong Kong and Vietnam (Adams *et al.*, 1997; Farci & Purcell, 2000). The presence of multiple genotypes may reflect the long-term evolution of viruses in discrete geographical areas (Bukh *et al.*, 1995).

Extensive studies have been done to understand the association of viral genotype with pathogenesis, disease severity and treatment responses. Infection with all currently recognised HCV genotypes is hepatotropic and pathogenic. Patients infected with genotype 1 (in particular 1b) and genotype 4 viruses respond poorly to IFN-α and ribavirin, achieving sustained clearance rates of only 50 %, whereas more than 75 % of patients infected with viruses of genotypes 2 and 3 achieve a sustained virologic response (Farci & Purcell, 2000; Hadziyannis & Koskinas, 2004; Zeuzem *et al.*, 2004). In addition, infection with genotype 3 viruses is associated with more frequent spontaneous viral clearance (Lehmann *et al.*, 2004). Infection with genotype 3 is also associated with development of steatosis (Rubbia-Brandt *et al.*, 2001). Zignego *et al.* reported that infection with genotype 2a viruses is more prevalent in patients with mixed cryoglobulinemia (Monteverde *et al.*, 1997; Zignego *et al.*, 1996). Some studies also suggest that different types of HCV may be associated with different transmission routes – e.g. genotype 3a appears to be prevalent among intravenous drug users.

Another important aspect of HCV infection is the heterogeneity of viral isolates within individuals. As is characteristic of many RNA viruses, high level replication and the

error-prone nature and lack of proofreading capacity of the viral RNA polymerase enable HCV to accumulate mutations rapidly. This results in circulation of HCV as quasispecies, which can differ genomically by as much as 1-5 %. The dominant viral species can change over time, as different selective pressures (e.g. host immune responses, drug treatments) are imposed upon the circulating quasispecies. The presence of the quasispecies may thus be an important mechanism used by HCV to evade host immune responses and to establish persistent infections.

1.2.4. Molecular clones

The initial cloning of HCV was done using high titered pools of chimpanzee serum as a source of virus. Molecular clones of HCV were developed from distinct cDNA clones derived from HCV genomic RNA, and their infectivity was demonstrated using the chimpanzee as a host. A number of full-length genotype 1a clones were derived from the Hutchinson H77 isolate of HCV (Feinstone *et al.*, 1981), however these clones exhibited slight variation in aa sequence. Yanagi *et al.* constructed a consensus HCV 1a clone called H77c (Yanagi *et al.*, 1997). Synthetic full-length transcripts generated from this clone were directly inoculated into the liver of a chimpanzee and the animal developed an infection characteristic of HCV (Yanagi *et al.*, 1999). Hong *et al.* also constructed a consensus HCV 1a clone called HCV-H that was derived from previously cloned sequences of the H77 isolate (Hong *et al.*, 1999). They demonstrated the infectivity of this clone, and further showed that injection of serum from the infected chimpanzee into a second animal also caused infection with HCV, indicating that molecular clones can generate transmissible infectious virions. Molecular clones from other HCV subtypes and genotypes are also available: genotype 1b (Beard *et al.*, 1999; Yanagi *et al.*, 1998) and genotype 2a (Yanagi *et al.*, 1999).

The development of molecular clones has enabled studies to characterise virological properties in animals infected with well-defined HCV inocula and analysis of genetic diversification during the course of progressive infection.

1.2.5. Animal model systems

Currently, chimpanzees are the only species other than humans highly susceptible to infection with HCV and this animal model has contributed much to HCV research over the past 20 years. The chimpanzee model has been used to characterise the natural history of infection, routes of transmission, the role of immune responses and the clinical outcomes of infection. In addition, these animals were also used to characterise the infectious nature of molecular clones. The use of chimpanzees permits the induction of infection with well-characterised molecular clones and sampling throughout the course of infection, including at early time-points, enabling studies into the mechanisms of viral clearance.

Chimpanzees are 98.5 % genetically identical to humans and undergo a similar clinical course of infection with HCV, with some animals developing a persistent infection with liver damage. However, persistent infection occurs in only 30-40 % of chimpanzees, while the rate of viral persistence in human is much higher (Lanford *et al.*, 2001). It is important to consider that the chimpanzee model represents a truly unselected population, while in human studies the individuals analysed are selected on the basis of disease status and antibody (Ab) response to HCV proteins. Moreover, chimpanzees exhibit a lesser degree of liver damage compared to humans, and they are less likely develop HCC (Bradley *et al.*, 1981; Muchmore *et al.*, 1988). However, the chimpanzee model also has disadvantages; chimpanzees have been listed as an endangered species since 1988, so are rare and expensive; they are also difficult to handle, and require

appropriate research facilities with proper surgical support and specialized veterinary care.

Another potential animal model for HCV infection is the tree shrew *Tupaia belangeri chinensis* (Xie *et al.*, 1998). Although these animals have been reported to support HCV replication, only 25 % of the animals inoculated with HCV were found to develop viraemia, Abs against HCV and elevated levels of ALT (Xie *et al.*, 1998). Moreover, since the initial report of Tupaia infection by HCV, only one study utilising this model has been published (Barth *et al.*, 2005).

Recently, immunodeficient mice into which human or chimpanzee hepatocytes are transplanted have been described (Maeda *et al.*, 2004; Mercer *et al.*, 2001). These mice can support HCV replication and generate transmissible infectious virions, but the development of liver disease is yet to be confirmed. These mice may be a useful tool for evaluation of antiviral therapies and potential vaccine candidates. However, use of such mice is limited by the requirement for human hepatocytes for their production, the special expertise needed to transplant them and the high mortality rate that these animals exhibit.

1.3. Pathogenesis of HCV infection

1.3.1. Transmission

HCV is a blood-borne virus and is transmitted through direct blood contact. After a successful HCV screening scheme introduced in 1990 reduced the transmission rate via blood transfusion and blood products, the major route of transmission became via

sharing of contaminated needles by intravenous drug users. Intravenous drug users are at a high risk of becoming HCV infected, with prevalence rates of 90 % amongst long-term users (Cramp *et al.*, 1999). Vertical and horizontal transmission are also possible, but are inefficient (Cohen, 1999; Memon & Memon, 2002). Thus the risk factors for HCV infection include intravenous drug use, haemodialysis, transfusion of blood and blood products, tattooing, acupuncture, high risk sexual behaviour, occupational exposure in healthcare workers and receipt of organ transplants from HCV-positive donors (Memon & Memon, 2002).

1.3.2. Tropism

The liver is the main site of HCV replication and it has been demonstrated that viral replication takes places in hepatocytes in HCV-infected patients (Blight *et al.*, 1994).
In addition, several lines of evidence support HCV replication at extrahepatic sites. Firstly, the presence of HCV positive sense RNA and replicative intermediates has been reported in some peripheral blood mononuclear cell (PBMC) subsets and $CD34^+$ hematopoietic progenitor cells (Bouffard *et al.*, 1992; Goutagny *et al.*, 2003; Lerat *et al.*, 1998; Muller *et al.*, 1993; Zignego *et al.*, 1992). Viral sequences have been detected in polymorphonuclear leukocytes, monocytes, macrophages, dendritic cells (DCs) and B cells (Ducoulombier *et al.*, 2004; Goutagny *et al.*, 2003; Lerat *et al.*, 1998), but not $CD4^+/CD8^+$ T cells or natural killer (NK) cells (Lerat *et al.*, 1998; Muller *et al.*, 1993). However, the detection of negative-stranded RNA in antigen presenting cells (APCs) and B cells does not fully prove the active replication of virus in these cells, as APCs may have taken up other cells in which HCV replication was ongoing. Opsonised viral particles can also be taken up by both phagocytic cells and B cells via Fc receptors. HCV proteins (core or NS5A) have also been detected in PBMCs (Gong *et al.*, 2003;

Hofmann *et al.*, 2004) and Bouffard *et al.* found HCV core epitopes in monocytes (Bouffard *et al.*, 1992). No correlation has been found between the presence of HCV RNA in PBMCs and viral genotypes or viral load (Lerat *et al.*, 1998).

Secondly, distinct glycoprotein sequence variants have been reported to be present in the liver and PBMCs (Laskus *et al.*, 2000; Okuda *et al.*, 1999); and in B cells and monocytes (Ducoulombier *et al.*, 2004), supporting the idea that HCV replicates in both liver and PBMCs and evolves independently in each compartment due to distinct immune selection pressures.

Thirdly, it is suggested that PBMCs may act as a reservoir of viruses because of the recurrence of hepatic HCV infection after liver transplantation (Botero, 2004; Dahari *et al.*, 2005). In addition, HCV infection is frequently associated with autoimmune extrahepatic manifestations (Ferri & Zignego, 2000), although the pathogenesis of these diseases is not fully understood.

It is unclear at which stage of infection PBMCs start to harbour HCV. Muller *et al.* found that only chronically infected patients carried HCV RNA in PBMCs (Muller *et al.*, 1993). Notably, Radkowski and Laskus reported that HCV RNA, including replicative intermediate forms of the genome, can persist in PBMCs for many years even after complete spontaneous or antiviral-therapy-induced resolution of chronic hepatitis C (Radkowski & Laskus, 2005).

1.3.3. Course of infection and associated diseases

Acute hepatitis C infection is usually asymptomatic, thus diagnosis is very rare at this stage. Chronic hepatitis C infection is also frequently asymptomatic for years or many decades, resulting in diagnosis of the infection being made at a late stage. HCV infection accounts for 70 % of all chronic hepatitis cases (Alter, 1997). Chronic

hepatitis C infection is also a major cause of cirrhosis and HCC. Importantly, end-stage liver disease secondary to HCV infection is the leading cause of liver transplantation (Botero, 2004). A hallmark of HCV infection is its high rate of chronicity, potentially producing a large population of HCV carriers.

1.3.3.1. Acute infection

After an initial incubation period, the virus undergoes a burst of replication, resulting in the presence of HCV RNA in the serum, which can be detected by polymerase chain reaction (PCR), within 1-2 weeks after exposure (Thimme *et al.*, 2001). The presence of viral RNA in the serum constitutes the earliest sign of HCV infection. In some infected individuals, HCV RNA can be detected in the serum but in other individuals it takes much longer before viraemia is detected (Farci *et al.*, 2000). Likewise viraemia can peak 4-6 weeks after exposure, but in some infected individuals, peak viraemia does not occur until 3-4 months post-infection (Farci *et al.*, 2000). Peak serum RNA levels are typically in the range of 10^6-10^8 genomes/ml, and occur before the peak in serum ALT levels and onset of symptoms (Alter *et al.*, 1995; Thimme *et al.*, 2001). ALT levels, representing hepatocyte injury and necrosis, start to rise 2-12 weeks after exposure, and may reach peak levels more than 10-fold higher than normal (Hoofnagle, 2002; Thimme *et al.*, 2001). In HCV infection, humoral immune responses are delayed until at least 7-12 weeks after exposure, and cellular immune responses are delayed by at least one month (Farci *et al.*, 2000; Thimme *et al.*, 2001). The acute phase of HCV infection is frequently asymptomatic and thus often goes unnoticed; but some infected individuals (~20 %) become icteric, which occurs on average after a 7-8 week incubation period (Marcellin, 1999). Acute hepatitis can result in fulminant hepatitis, but this is very rare (Hoofnagle *et al.*, 1995). In patients with self-limited hepatitis, within a few weeks of

onset of symptoms, HCV RNA becomes undetectable and ALT levels return to normal; anti-HCV Abs progressively decrease but remain detectable for many years (Marcellin, 1999). Interestingly, patients who are highly symptomatic in the acute phase have a higher chance of recovering than asymptomatic patients (Gerlach *et al.*, 2003). Moreover, it has been reported that patients in whom rapid virus replication occurs show stronger cytotoxic T lymphocyte (CTL) responses, which likely results in the resolution of hepatitis in the acute phase (Bocharov *et al.*, 2004b).

1.3.3.2. Chronic infection

Only around 20 % of infected individuals clear the virus spontaneously after acute infection; the rest develop a persistent infection characterised by the presence of continuous virus replication. Once chronic infection is established, serum HCV RNA levels stabilise (Yeo *et al.*, 2001) and anti-HCV Ab titres increase to higher levels. Approximately one third of chronic HCV patients have persistently normal serum ALT levels despite of the presence of HCV RNA in serum, whereas others have intermittently elevated serum ALT levels (Conry-Cantilena *et al.*, 1996). The patients with normal levels of ALT usually have a good prognosis.

Many patients with chronic hepatitis C have few symptoms, and these are usually non-specific, intermittent and mild. The most common symptom is fatigue (Hoofnagle, 1997). However, chronic infection is associated with a risk of progressive liver disease and the development of cirrhosis and HCC. Nearly all patients who develop persistent viraemia, with or without increasing levels of ALT, have histological changes on liver biopsy, but the severity of disease and the amount of structural damage (fibrosis) varies considerably. It is thought that 20-35 % of chronic HCV patients will eventually develop cirrhosis (Shakil *et al.*, 1995); and 1-5 % of the patients with cirrhosis will

progress to HCC. Disease progression is very slow: on average, it takes 20.6 years to develop cirrhosis and 28.3 years to develop HCC (Tong *et al.*, 1995). However other factors, such as co-infection with hepatitis B virus (HBV) or human immunodeficiency virus (HIV), older age, immunodeficiency or alcohol intake can accelerate the progression of disease. Virologic factors such as viral load and the HCV genotype have not been found to affect the progression of disease (Zeuzem *et al.*, 1996). Liver transplantation is the only way to extend the survival of patients with end-stage liver disease; however recurrent HCV infection of the new allograft is universal and is frequently associated with more rapid progression to cirrhosis and end-stage disease (Feray *et al.*, 1992).

The mechanisms underlying the liver damage and HCC associated with HCV infection remain poorly understood. A key issue is whether the liver disease is caused by direct cytopathic effects of the virus itself or as a consequence of the immune response it induces.

1.3.3.3. Extrahepatic manifestations

HCV infection is also suggested to play a crucial role in autoimmune and lymphoproliferative disorders (Ferri & Zignego, 2000). The disease which is most clearly and most often associated with HCV is mixed cryoglobulinemia (type II and type III) (Agnello *et al.*, 1992). 80-100 % of patients with mixed cryoglobulinemia are infected with HCV (Ferri *et al.*, 1998; Ferri *et al.*, 1991; Misiani *et al.*, 1992). Mixed cryoglobulinemia is a lymphoproliferative disorder characterised by stimulation and monoclonal or polyclonal expansion of B cells in the blood, bone marrow, liver and spleen, leading to the production of large amounts of rheumatoid factors and temperature-sensitive circulating immune complexes that accumulate in different tissues

(Zignego *et al.*, 1995). Type II and III cryoglobulinemia are characterised by IgG and monoclonal or polyclonal IgM with rheumatoid factor activity respectively (Ferri & Zignego, 2000). The cryoglobulins found in the serum of HCV-infected patients are composed of immune complexes of HCV, Abs to HCV, immunoglobulins, rheumatoid factor and complement (Marcellin, 1999). Almost half the IgG possesses anti-HCV activity (Szymanski *et al.*, 1994) and a much higher concentration of viral genome was found in the cryoprecipitate than in the supernatant (Agnello *et al.*, 1992; Ballare *et al.*, 1993).

Although the majority of mixed cryoglobulinemia cases are asymptomatic (Marcellin, 1999), the deposition of circulating immune complexes and complement can cause systemic vasculitis of small to medium-sized vessels and glomerulonephritis (Ferri & Zignego, 2000; Stehman-Breen *et al.*, 1995).

Despite the fact that the majority of patients with mixed cryoglobulinemia are infected with HCV, monoclonal expansion and proliferation of B cells leading to mixed cryoglobulinemia occurs only in a small subset of HCV-infected patients (Gerotto *et al.*, 2001; Marcellin, 1999; Pawlotsky *et al.*, 1994). The increased frequency of B cells observed in some chronic HCV patients is not correlated with the development of mixed cryoglobulinemia (Ni *et al.*, 2003). It is reported that infection with genotype 2a HCV is particularly associated with the development of type II mixed cryoglobulinemia (Monteverde *et al.*, 1997; Zignego *et al.*, 1996). The geographical heterogeneity observed in the prevalence of HCV-related mixed cryoglobulinemia (Ferri & Zignego, 2000; Ferri *et al.*, 1998; Lenzi *et al.*, 1991) indicates that the genotype of HCV and/or genetic/environmental factors contribute to the development of mixed cryoglobulinemia in HCV-infected patients.

In addition to mixed cryoglobulinemia, other autoimmune lymphoproliferative disorders have been reported in HCV infection. Indeed, 70 % of HCV-infected patients have circulating autoreactive Abs (Cacoub *et al.*, 2000). HCV-related disorders include porphyria cutanea tarda, autoimmune hepatitis, membranoproliferative glomerulonephritis, B cell neoplasias and non-Hodgkin's lymphoma (Ferri *et al.*, 2002; Ferri *et al.*, 1993; Ferri *et al.*, 1994; Gisbert *et al.*, 2003). Striking geographical and racial variation in these associations suggests that genetic and/or environmental factors may also play a role in the pathogenesis of these disorders.

1.4. HCV E2 glycoprotein

1.4.1. Biogenesis of HCV envelope glycoproteins

Enveloped viruses possess a lipid bilayer derived from the host, containing viral proteins. The envelope proteins play important roles in the viral life-cycle, which include packaging of viral proteins into an infectious particle, mediating the attachment and entry of virions into host cells through interaction with receptor(s) expressed on the target host cells and inducing fusion between the host cell membrane and viral envelope. HCV possesses two predicted envelope surface glycoproteins, E1 and E2. Both glycoproteins (E1 ~31 KDa and E2 ~70 KDa) are believed to be type I integral transmembrane proteins with a large N-terminal ectodomain and a C-terminal hydrophobic anchor domain (Rice, 1996). The ectodomains of the glycoproteins are targeted to the ER lumen where they are heavily modified by N-linked glycosylation (Dubuisson *et al.*, 1994; Goffard & Dubuisson, 2003). A putative model of the biogenesis of the HCV glycoproteins is summarised in Fig. 1.3.

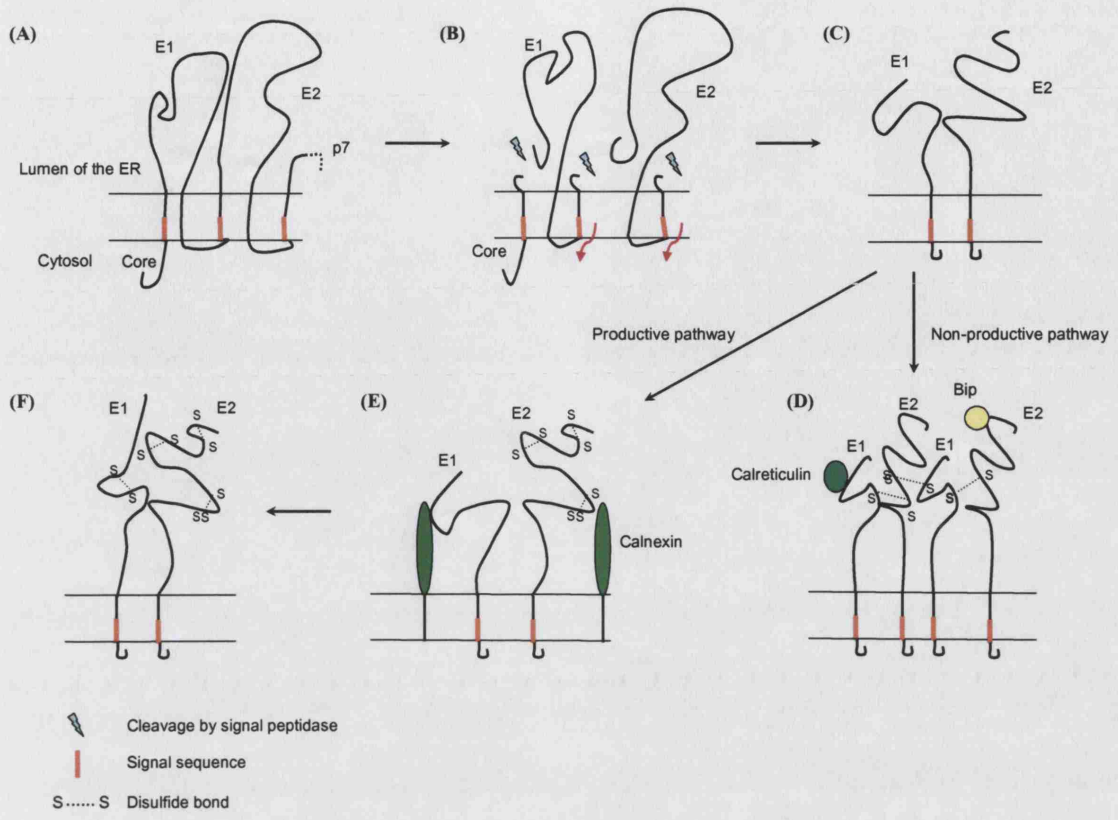


Figure 1.3. Putative model summarising the biogenesis of the HCV glycoproteins.

The HCV polyprotein is synthesised at the endoplasmic reticulum (ER) (A). Signal peptidase localised on the lumenal side of the ER mediates cleavages at the N-termini of the E1, E2 and p7 proteins (B). After the cleavage, reorientation of the transmembrane domains of the glycoproteins occurs, leaving the C-termini of E1 and E2 in the cytosol and the N-termini in the lumen of the ER (C). Association of the glycoproteins with the ER chaperones calreticulin and Bip leads to a non-productive folding pathway characterised by the formation of intermolecular disulfide bonds and generation of aggregates of E1 and E2 (D). On the other hand, association of E1 and E2 with calnexin leads to the formation of intramolecular disulfide bonds firstly within E2 (E) and then within E1 (F). This constitutes a productive folding pathway, resulting in the formation of a non-covalently linked heterodimer of E1 and E2 (F). (Figure adapted from Dubuisson *et al.*, (2000), Op De Beeck *et al.*, (2001) and Dubuisson *et al.*, (2002))

During HCV protein synthesis, all proteins are expressed as a single polyprotein at the ER with the transmembrane domains inserted into the membrane of the ER and the ectodomains of the glycoproteins targeted to the ER lumen. (Fig. 1.3-A). The C-termini of the core and E1 proteins contain signal sequences within their transmembrane domains that direct the translocation of the ectodomains of E1 and E2 into the ER lumen (Cocquerel *et al.*, 2002; Santolini *et al.*, 1994). Before cleavage, the transmembrane domains of both glycoproteins form a hairpin structure, which contains two transmembrane spanning sites (Fig. 1.3-A). The cleavage of the structural proteins is mediated by a host signal peptidase localised in the ER (Fig. 1.3-B) (Reed & Rice, 2000). The cleavages at the signal sequences within the transmembrane regions of the core and E1 proteins are completed rapidly after translation (Dubuisson, 2000; Dubuisson *et al.*, 1994). However, the cleavage at the E2/p7 junction is delayed, and is incomplete for at least the H77 strain, which results in the production of fully processed E2 and uncleaved E2/p7 (Lin *et al.*, 1994). The functions of the two different forms of H77c E2 are yet to be determined.

After the cleavage, the orientation of the C-termini of the transmembrane domains of both glycoproteins changes from the lumenal side of the ER to the cytosolic side, leaving each protein with a single membrane-spanning topology (Fig. 1.3-B and -C) (Cocquerel *et al.*, 2002; Op De Beeck *et al.*, 2000). This leads to interaction of the transmembrane domains of the E1 and E2 glycoproteins. The transmembrane domains are reported to be essential in the formation of E1E2 heterodimers (Cocquerel *et al.*, 1998; Michalak *et al.*, 1997; Patel *et al.*, 2001). After synthesis and cleavage, the glycoproteins accumulate within the ER due to the presence of ER retention signals at the C-termini of both E1 and E2 (Cocquerel *et al.*, 2000; Cocquerel *et al.*, 2002). The

middle of the transmembrane domains of the E1 and E2 proteins contains one or several highly conserved hydrophilic residues, which are responsible for the retention of glycoproteins in the ER. Thus the transmembrane domains of the HCV glycoproteins have multiple functions: (1) membrane anchoring; (2) E1E2 heterodimerisation; and (3) ER retention (Cocquerel *et al.*, 2002).

When expressed *in vitro*, two forms of E1 and E2 are observed: non-covalent heterodimers, which are assumed to be a native pre-budding form and heterogenous disulfide-linked aggregates (Dubuisson *et al.*, 1994). The latter are misfolded dead-end protein products resulting from a defective folding pathway and are thought to be generated as a result of high level protein expression in *in vitro* systems. In support of this, conformation-dependent monoclonal antibodies (mAbs) can recognise only non-covalent forms of E2 but not aggregates (Deleersnyder *et al.*, 1997; Michalak *et al.*, 1997). However, Liberman *et al.* reported that E2 can transactivate the genes encoding the ER chaperones Bip (GRP78) and GRP94, and bind tightly to Bip (Liberman *et al.*, 1999), implying the aggregates may have a role *in vivo* (discussed in detail later). Moreover, it is hypothesised that the presence of aggregates in the non-productive pathway may play a role in the down-regulation of particle formation to minimize exposure of viral antigens to the immune system and/or reduce pathogenesis (Dubuisson, 2000). Due to the lack (until recently) of a cell culture system supporting HCV replication *in vitro*, it has not yet been determined whether aggregates are produced in the authentic HCV lifecycle.

Correct folding of glycoproteins is partially assisted by the presence of the ER chaperone proteins calnexin, calreticulin and Bip (Choukhi *et al.*, 1998; Dubuisson &

Rice, 1996). The fate of E1E2 heterodimers seems to be dependent on the ER chaperone proteins they interact with. It is known that calreticulin and Bip interact preferentially with aggregates of E1 and E2, while calnexin interacts with functional non-covalently linked E1 and E2 (Choukhi *et al.*, 1998). In the non-productive pathway, glycoproteins are associated with calreticulin and Bip, leading to the formation of intermolecular disulfide bonding (Fig. 1.3-D). This results in the formation of aggregates. Conversely, the interaction of E1 and E2 with calnexin assists intramolecular disulfide bond formation, which results in the non-covalent E1E2 heterodimer formation in the productive pathway (Fig. 1.3-E).

Retention of glycoproteins in the ER plays an important role in the biogenesis of proteins. The lumen of the ER provides an environment that helps proteins acquire the correct cysteine pairing during the folding process. HCV glycoproteins contain highly conserved cysteine residues, implying that E1 and E2 acquire cysteine pairs and fold into a functional conformation in the ER.

In addition, the lumen of the ER provides enzymes that catalyse the glycosylation of proteins. HCV contains up to 5 and 11 potential N-linked glycosylation sites in E1 and E2 respectively. The glycans play a major role in the folding of these proteins, because the absence of glycans on E1 and E2 leads to misfolding.

Interestingly, it is known that proper folding of E2 does not require E1, whereas folding of E1 is dependent on the presence of E2 (Michalak *et al.*, 1997). Supporting this, disulfide bond formation of E1 occurs after E2 acquires disulfide bonding (Dubuisson, 2000), and the glycosylation of E1 is also improved by the presence of E2 (Dubuisson *et al.*, 2000).

HCV particles are presumed to bud from the ER due to the presence of the ER retention signals in the glycoproteins (Bartenschlager & Lohmann, 2000; Dubuisson *et al.*, 2002). It is hypothesised by analogy with other viruses that HCV might be secreted via the host cell secretory pathway (Mackenzie & Westaway, 2001). Thus, while particles move from the ER to the extracellular environment, they pass through the Golgi apparatus, enabling modification of the glycans on the viral glycoproteins by Golgi enzymes (Sato *et al.*, 1993).

1.4.2. Interaction of E2 with host cell surface proteins

Understanding the interaction of E2 with host cell surface receptors has been facilitated by the development of recombinant E2 proteins. Initially, it was found that truncation of E2 to remove the hydrophobic transmembrane domain and C-terminal region resulted in secretion of a soluble form of E2 which was folded in a manner comparable to E2 in the E1E2 complex (Flint *et al.*, 1999b; Matsuura *et al.*, 1994; Michalak *et al.*, 1997). The truncated, soluble E2 has proved to be a useful tool for studying E2 binding to host cells; however, it has to be considered that its behaviour may not entirely reflect that of native E2 in a heterodimer with E1. Recently, several surrogate models of HCV virions have been developed to allow investigation of the interaction of HCV with host cells. They include HCV virus-like particles (VLPs) generated using baculovirus expression systems (Baumert *et al.*, 1998; Clayton *et al.*, 2002; Steinmann *et al.*, 2004; Triyatni *et al.*, 2002a; Triyatni *et al.*, 2002b), pseudotyped vesicular stomatitis virus (VSV) (Matsuura *et al.*, 2001) and pseudotyped retroviral particles (Bartosch *et al.*, 2003a; Flint *et al.*, 2004; Op De Beeck *et al.*, 2004). These model particles express

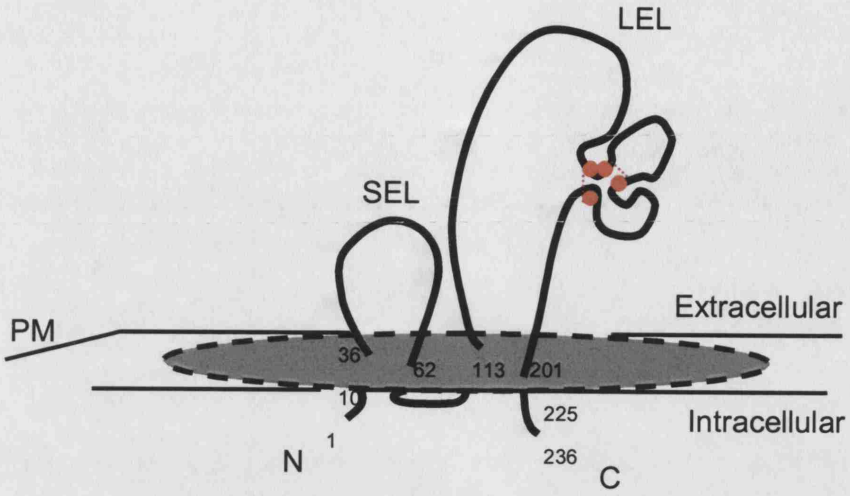
heterodimers of E1E2 in an array on the virion surface that may be more representative of the native form of E2.

Several cell surface molecules have been reported to bind E2 glycoproteins: CD81 (Pileri *et al.*, 1998); scavenger receptor class B type I (SR-BI) (Scarselli *et al.*, 2002); the low-density lipoprotein (LDL) receptor (Agnello *et al.*, 1999; Wunschmann *et al.*, 2000); dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) and liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin (L-SIGN or DC-SIGNR) (Gardner *et al.*, 2003; Lozach *et al.*, 2003; Pohlmann *et al.*, 2003); and glycosaminoglycans (GAGs) (Basu *et al.*, 2004; Takikawa *et al.*, 2000; Yagnik *et al.*, 2000). Among these, DC-SIGN, L-SIGN and GAGs have been suggested to function as capture receptors that may facilitate HCV interaction with specific entry receptors (Basu *et al.*, 2004; Gardner *et al.*, 2003; Lozach *et al.*, 2004; Pohlmann *et al.*, 2003), whereas CD81 and SR-BI are thought to act as co-receptors that mediate HCV binding and subsequently cell entry and infection (Bartosch *et al.*, 2003b; Zhang *et al.*, 2004).

1.4.2.1. E2 binding to CD81

CD81 is a member of the tetraspanin superfamily of proteins characterised by possessing four transmembrane domains and two extracellular domains (described in detail later)(Levy *et al.*, 1998). Transmembrane domains 1 and 2 flank a small extracellular loop (SEL), whereas transmembrane domains 3 and 4 flank a large extracellular loop (LEL) (Fig. 1.4-A). It has been reported that both soluble truncated E2 and full-length E2 in the form of heterodimers with E1 bind to CD81 (Cocquerel *et al.*, 2003; Owsianka *et al.*, 2001; Pileri *et al.*, 1998; Zhang *et al.*, 2004). It has been

(A)


LEL
SEL
Extracellular
PM
36
62
113
201
10
225
Intracellular
1
N
236
C

(B)

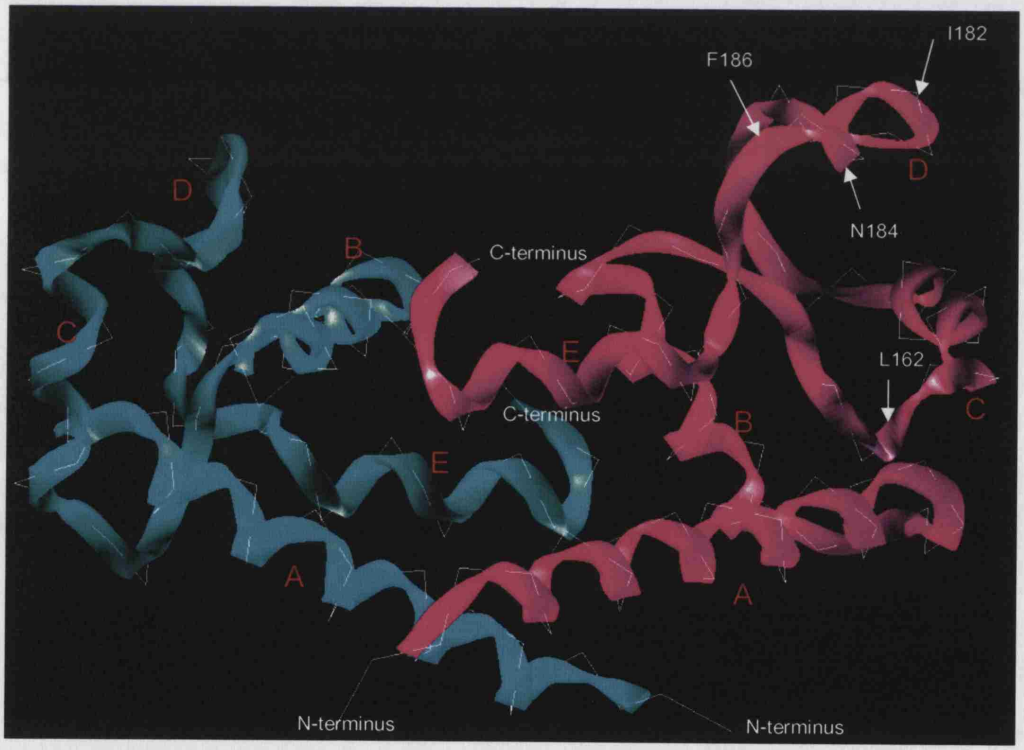

I182
F186
D
N184
B
C-terminus
C
E
L162
C-terminus
B
C
E
A
A
N-terminus
N-terminus

Figure 1.4. Structure of CD81.

(A) Schematic structure of CD81 (Figure adapted from Levy and Shoham, (2005)). CD81 is a transmembrane protein possessing four transmembrane domains embedded in the plasma membrane (PM). CD81 contains short cytoplasmic domains at the amino (N) and carboxyl (C) termini. Transmembrane domains 1 and 2 flank a small extracellular loop (SEL), whereas transmembrane domains 3 and 4 flank a large extracellular loop (LEL). The orange circles represent key cysteine residues in the LEL at amino acids (aa) 156, 157, 175 and 190. The cysteine residues at positions 156 and 157 form the distinct Cys-Cys-Gly motif. The numbers of the first and last aa residues of the cytoplasmic domains, SEL and LEL are indicated. (B) Ribbon diagram of a CD81-LEL dimer. The structure of the LEL homodimer is taken from the RCSB protein data bank, accession number 1G8Q (Kitadokoro et al., (2001)). The LEL of CD81 consists of five α-helices (A-E). The A and E domains form a stalk structure, whereas the B, C and D domains comprise the head subdomain. The A-, B- and E-helices are suggested to play a role in CD81-CD81 homodimerisation. The C- and D-helices are solvent-exposed regions and are suggested to be a crucial site for functional protein-protein interactions. The two monomers are shown in pink and light blue colours. The N and C termini are indicated. The five helices (A-E) are labelled in red. The E2 binding sites (leucine (L) at aa 162, isoleucine (I) at aa 182, asparagine (N) at aa 184 and phenylalanine (F) at aa 186) identified by Drummer et al., (2002) are all located in the C and D domains and their locations are shown by arrows.

shown that the LEL of CD81 is sufficient for E2-CD81 interaction, and that no additional human cell specific factors are required for the primary interaction (Pileri *et al.*, 1998).

Although CD81 has been suggested to be a host cell receptor for HCV, many lines of evidence suggest that CD81 may not directly mediate HCV infection of susceptible cells. Firstly, E2 was observed to bind not only to CD81 of humans and chimpanzees (Flint *et al.*, 1999b), but also tamarins, a group of New World monkeys that are not susceptible to HCV infection (Allander *et al.*, 2000; Meola *et al.*, 2000). This indicates that E2-CD81 interaction does not predict susceptibility to HCV infection. Secondly, most human tissues express CD81 except for red blood cells and platelets (Engel & Tedder, 1994; Levy *et al.*, 1998), thus E2-CD81 interaction does not explain the hepatocyte and APC-specific tropism of HCV. Thirdly, CD81 is reported to mediate internalisation poorly in hepatocytes (Petracca *et al.*, 2000; Rice, 1999; Takikawa *et al.*, 2000). Fourthly, some hepatoma cell lines can bind HCV glycoproteins despite lacking CD81 expression (Petracca *et al.*, 2000). Finally, expression of CD81 does not confer HCV susceptibility on non-hepatic or non-permissive cells (Bartosch *et al.*, 2003a; Bartosch *et al.*, 2003b; Hsu *et al.*, 2003; Zhang *et al.*, 2004) and transgenic mice expressing human CD81 are not susceptible to HCV infection (Masciopinto *et al.*, 2002). These results all suggest that additional or alternative host cell receptors exist. Nonetheless, it is strongly believed that E2-CD81 interaction is required for HCV binding and entry into host cells, functioning to concentrate viral particles for effective entry, or acting as a co-receptor (Bartosch *et al.*, 2003b; Hsu *et al.*, 2003; Lavillette *et al.*, 2005a; McKeating *et al.*, 2004; Zhang *et al.*, 2004).

Since CD81 is likely to be a part of the receptor complex involved in mediating HCV infection, many studies have focused on the identification of E2 binding sites on the LEL of CD81 to enable the design of antiviral drugs and vaccines. In addition, as discussed later, E2-CD81 interaction has been shown to be able to modulate immune cell functions. Thus characterisation of the E2 binding site on CD81 may give insight into the structure and the functions of CD81. The crystal structure of the LEL revealed that it forms a homodimer, with each monomer containing five α-helices (A-E) in an arrangement of stalk and head subdomains (Fig. 1.4-B) (Kitadokoro *et al.*, 2001). Two main aspects of the LEL structure have to be considered in E2-LEL interaction: (1) epitopes in the head subdomain (in the C and D helix regions) which are required for E2 binding; and (2) the intramolecular structure maintained by disulfide bonding.

Comparison of the CD81 sequences from human and African green monkey (the latter does not bind to E2) revealed that they differ at only 4 aa residues, at aa positions 163, 186, 188 and 196 (Pileri *et al.*, 1998). Although phenylalanine (F) at position 186 in the LEL was found to be essential for binding of soluble truncated E2 to CD81 (Higginbottom *et al.*, 2000; Meola *et al.*, 2000), HCV pseudotyped particles were found to infect hepatic cells expressing CD81 with a phenylalanine to leucine substitution at position 186 at an equivalent level to cells with normal CD81 (Zhang *et al.*, 2004). Studies using random mutagenesis identified aa 182, 184, 186 and 162 as being of importance for E2 binding to the LEL, as mutations at these residues abolished E2 binding (Drummer *et al.*, 2002). Residues 182, 184 and 186 form a part of a cluster comprising a hydrophobic ridge that is located on an exposed site in the head subdomain of the LEL (Drummer *et al.*, 2002; Kitadokoro *et al.*, 2001). This explains the observation made by Zhang *et al.*, (2004) that E2 recognises a region comprising phenylalanine at position 186 and surrounding amino acids in the LEL. Amino acid 163

is also known to be exposed on the surface of the head subdomain (Kitadokoro *et al.*, 2001) and is suggested to contribute to E2-CD81 interaction by maintaining the tertiary structure of CD81 (Higginbottom *et al.*, 2000). In addition, the aa residue at position 162 is suggested to be part of an epitope recognised by the anti-CD81 mAb clone 1.3.3.22, which blocks E2-CD81 binding (Drummer *et al.*, 2002).

In addition to the epitopes mentioned above, the conformation of CD81 is also essential in E2-CD81 interaction, as heat denaturation breaks the interaction and reduction of disulfide bonding within the LEL impairs E2 binding (Flint *et al.*, 1999a; Higginbottom *et al.*, 2000; Petracca *et al.*, 2000). The distinct CCG motif and two other cysteine residues present in the LEL are highly conserved throughout tetraspanin family members, forming two intramolecular disulfide bonds (Fig. 1.4-A) (Kitadokoro *et al.*, 2001). Cysteine residues at aa 157 and 190 have been shown to be essential for folding of the LEL and E2 binding (Drummer *et al.*, 2005). In addition, Drummer *et al.* suggested that domains from both inside and outside the LEL contribute to CD81 dimer stability, thus structural differences between dimers of the isolated LEL and intact CD81 must be taken in account when CD81-E2 interaction is studied (Drummer *et al.*, 2005).

1.4.2.2. E2 binding to SR-BI

Human SR-BI (alternatively known as CLA-1) is a member of the CD36 superfamily, possessing a horseshoe-like structure with one large extracellular loop and short N- and C-terminal cytoplasmic domains (Krieger, 2001). SR-BI is expressed primarily in the liver and steroidogenic tissues, but also on monocytes and macrophages, astrocytes and vascular smooth muscle cells in the brain (Chinetti *et al.*, 2000; Husemann & Silverstein, 2001; Krieger, 1999). SR-BI functions as a high-density lipoprotein (HDL) receptor as

well as a LDL receptor to mediate the selective uptake of cholesterol (Acton *et al.*, 1996). In addition, SR-BI functions as a pattern-recognition receptor, particularly on monocytes and macrophages, recognising molecular patterns displayed on the surface of microorganisms or on apoptotic cells, and mediating phagocytosis of these molecules or cells for clearance (Bocharov *et al.*, 2004a; Imachi *et al.*, 2000; Murao *et al.*, 1997; Pearson, 1996; Vishnyakova *et al.*, 2003).

Scarselli *et al.* identified SR-BI as a novel E2 receptor (Scarselli *et al.*, 2002). It was shown that the E2 protein can bind to SR-BI through its hypervariable region (HVR)-1 (Bartosch *et al.*, 2003b; Scarselli *et al.*, 2002). Interestingly, it was also found that HDL can markedly enhance the entry of retroviral particles pseudotyped with HCV E1E2 into cells in a SR-BI-dependent manner (Bartosch *et al.*, 2005; Voisset *et al.*, 2005). Meunier *et al.* also found that apolipoproteins C1 can cause the same enhancement (Meunier *et al.*, 2005). Whilst HCV virions in the plasma of HCV-infected patients are frequently found in association with lipoproteins such as HDL and LDL (Agnello *et al.*, 1999; Andre *et al.*, 2002), there is no experimental evidence that HDL associates with HCV glycoproteins, implying that HCV does not use HDL as a carrier to bind to the receptors (Voisset *et al.*, 2005). It is speculated that HDL may be acting by stimulating enhanced internalisation of SR-BI.

Notably, although SR-BI plays a role in HCV binding and entry into host cells, expression of SR-BI alone or with CD81 failed to confer permissivity on cells to infection with HCV pseudotyped particles (Bartosch *et al.*, 2003b; Hsu *et al.*, 2003). This suggests that additional factor(s) also participate in the HCV attachment/entry process.

1.4.2.3. E2 binding to the LDL receptor

The LDL receptor has also been considered as a HCV receptor candidate, as several lines of evidence suggest that HCV virions in the plasma are associated with LDL and very low density lipoprotein (VLDL) (Agnello *et al.*, 1999; Thomssen *et al.*, 1992; Wunschmann *et al.*, 2000). The LDL receptor mediates cellular uptake of cholesterol through LDL. It is speculated that the association with LDL enables HCV virions to bind the LDL receptor by using the natural ligand (LDL) as a carrier; LDL-bound HCV can then be internalised into LDL receptor-expressing cells by endocytosis (Agnello *et al.*, 1999; Wunschmann *et al.*, 2000). However, it is not clear whether this pathway leads to productive HCV infection. Moreover, VLP binding to cells is not correlated with the expression of the LDL receptors (Wellnitz *et al.*, 2002), and there is no evidence that the LDL receptor is a major receptor for HCV pseudotyped particles (Bartosch *et al.*, 2003a; Hsu *et al.*, 2003). Moreover, the E2 protein does not mediate HCV particle-LDL receptor interaction and all forms of E1E2 fail to bind LDL (Lambot *et al.*, 2002; Wunschmann *et al.*, 2000). It is possible that LDL and VLDL cannot bind to mature HCV particles, but they may interact with HCV before or during virion assembly (Dubuisson *et al.*, 2002). It is also plausible that LDL may facilitate HCV virion uptake via LDL receptors in a similar manner to that by which HDL enhances virion uptake via SR-BI. Notably, it has been reported that the LDL receptor promotes entry of other members of the *Flaviviridae* family such as GB virus and BVDV (Agnello *et al.*, 1999).

1.4.2.4. E2 binding to DC-SIGN and DC-SIGNR

DC-SIGN is a mannose-binding C-type lectin receptor expressed on immature DCs in lymphoid and peripheral tissues and some types of tissue macrophages (Soilleux *et al.*,

2001; Soilleux *et al.*, 2002). In the peripheral blood, some $CD14^+$ cells express DC-SIGN, but myeloid and plasmacytoid DCs lack its expression (Engering *et al.*, 2002; Turville *et al.*, 2001). DC-SIGN is also highly expressed on immature monocyte-derived DCs (Baribaud *et al.*, 2002; Geijtenbeek *et al.*, 2000c), although expression is down-regulated when the cells mature (Ludwig *et al.*, 2004; Pohlmann *et al.*, 2003). DC-SIGNR or L-SIGN is a homologue of DC-SIGN. Although DC-SIGNR has the same ligand-binding specificity as DC-SIGN, its expression is restricted to lymph node sinuses and liver sinusoidal endothelial cells (Bashirova *et al.*, 2001; Cole *et al.*, 2004). The natural ligands for DC-SIGN are intercellular adhesion molecule (ICAM)-2 and ICAM-3 (Geijtenbeek *et al.*, 2000a; Geijtenbeek *et al.*, 2000b). ICAM-2-DC-SIGN interaction causes $DC\text{-}SIGN^+$ DCs to roll along the surface of the endothelium of blood vessels, helping transendothelial migration of DCs. Therefore the interaction may be crucial for recruitment of DCs to inflammatory sites and lymphoid tissues. In addition, DC-SIGN initiates the interaction of DCs with naïve T cells through ICAM-3, which is expressed on the surface of T cells. This interaction mediates close and stable DC-T cell contact, leading to successful T cell receptor (TCR) engagement.

HCV has been reported to bind to both DC-SIGN and DC-SIGNR via its E1 and E2 proteins (Gardner *et al.*, 2003; Lozach *et al.*, 2004; Lozach *et al.*, 2003; Ludwig *et al.*, 2004; Pohlmann *et al.*, 2003). In addition to HCV, DC-SIGN can bind to many other organisms including HIV (Geijtenbeek *et al.*, 2000c), cytomegalovirus (CMV) (Halary *et al.*, 2002), dengue virus (Tassaneetrithep *et al.*, 2003), *Schistosoma mansoni* (van Die *et al.*, 2003) and *Mycobacterium tuberculosis* (Geijtenbeek *et al.*, 2003). A common feature of the pathogens that interact with DC-SIGN is that they cause chronic infections (Appelmelk *et al.*, 2003). Although Ludwig *et al.* reported that VLPs can be internalised into monocyte-derived DCs in a DC-SIGN dependent manner (Ludwig *et*

al., 2004), DC-SIGN/DC-SIGNR did not mediate HCV pseudotyped particle infection of cells (Lozach *et al.*, 2004). However, infectious virus was transmitted efficiently in a DC-SIGN dependent manner when permissive Huh-7 cells were co-cultured with HCV pseudotyped particles bound to DC-SIGN/DC-SIGNR-positive cell lines (Lozach *et al.*, 2004). Interestingly, these particles were targeted to nonlysosomal compartments of early endosomes where they evaded lysosomal degradation, as seen in HIV-1 (Kwon *et al.*, 2002) and CMV infections (Halary *et al.*, 2002). These reports indicate that DC-SIGN and DC-SIGNR are not the main receptor for HCV, but it is plausible that HCV may utilise DC-SIGN/DC-SIGNR for enhancing cell attachment prior to interaction with more specific receptors, and for delivery of viruses to the liver, in a similar manner to that in which HIV-1 utilises DC-SIGN to enhance viral cell adhesion and transmission to T cells (Sol-Foulon *et al.*, 2002).

1.4.2.5. E2 binding to GAGs

GAGs are sulphated polysaccharide side-chains of cell surface proteoglycans (Bernfield *et al.*, 1999). Heparan sulphate is a type of GAG, which can be bound by many viruses including HCV (Yagnik *et al.*, 2000), dengue virus (Chen *et al.*, 1997b), HIV (Patel *et al.*, 1993) and herpes simplex virus (HSV) (Spear, 2004). GAGs are ubiquitously expressed on many types of cells and viruses use them as an initial attachment receptor that enables virions to be captured and brought into closer contact with more specific receptors which then mediate virus entry. It was shown that the HCV E2 protein can bind to GAGs through its HVR-1 (Basu *et al.*, 2004; Yagnik *et al.*, 2000), and that heparin and polyanionic compounds can inhibit HCV binding to PBMCs and hepatocyte cells (Cribier *et al.*, 1998; Garson *et al.*, 1999). However, the interaction between E2 and proteoglycans does not itself mediate HCV infection (Basu *et al.*, 2004). Thus

GAGs may play a role in facilitating virus-host interaction. It is thought that GAGs may be (at least one of) the additional factors that, together with CD81 and SR-BI, may mediate the HCV attachment/entry process.

1.5. Immune responses in HCV infection

Chronic HCV infection is associated with continuous viral replication *in vivo*. In most patients, the immune response fails to resolve the infection despite the presence of an innate response and adaptive humoral and cellular immune responses directed against both structural and NS proteins (Cerny & Chisari, 1994).

Studies of the HCV-specific immune response in humans and in chimpanzees experimentally infected with HCV have aimed to address differences in the immune response in individuals who resolve acute HCV infection and those who become chronically infected; why the immune response fails to control virus replication in the majority of cases; and whether or how the immune response in chronically-infected individuals may contribute to liver damage over time.

However, the factors determining the different outcomes of HCV infection are not clear. The establishment of chronic HCV infection is likely to be multifactorial process that is influenced by both host factors, such as the (in)ability of the host to mount a protective immune response, and viral factors, such as the degree of genetic heterogeneity of the virus.

1.5.1. Innate immunity

Innate immune responses are rapidly activated following viral infection, and typically play both a vital early effector role and a key role in the activation and shaping of

adaptive immune responses, which can determine the course of the infection (Biron & Brossay, 2001; Biron *et al.*, 2002; Medzhitov & Janeway, 1997). In addition, innate responses also participate in the containment of persistent viral infections (French *et al.*, 2004).

Although the adaptive responses during HCV infection have been studied intensively, innate immune responses remain poorly characterised. Nonetheless, it is suggested that innate immune responses are of critical importance for the clearance of HCV (Thomson *et al.*, 2003). It is plausible that some degree of defect is included in the innate immune response in the early phase of HCV infection, which results in inefficient antiviral immune responses at both the innate and adaptive level, and contributes to the high rate of chronicity of HCV infection.

1.5.1.1. Type I IFNs

Viral infection triggers host cells to release a variety of innate cytokines, most importantly type I IFNs (which include IFNs-α and -β). Many cell types can produce type I IFNs following viral infection, but the major source of type I IFN production in most virus infection is a specialised population of cells termed natural IFN producing cells or plasmacytoid DCs. These cells can be triggered to produce type I IFNs following recognition of pathogen components, without themselves being infected (Cella *et al.*, 1999; Siegal *et al.*, 1999). Type I IFNs have direct anti-viral effects, which are mediated through up-regulation of expression of genes encoding proteins with antiviral activity, e.g. 2'5' oligoadenylate synthetase, which is required for the activation of the endoribonuclease RNase L to degrade viral RNA, and double-stranded RNA dependent protein kinase R (PKR), which phosphorylates the translation initiation

factor eIF2α to inhibit protein synthesis (Samuel, 2001). In addition, type I IFNs play a pivotal role in modulating the activation, proliferation and differentiation of cells involved in both innate and acquired immunity (Biron, 2001; Le Bon & Tough, 2002). For example, IFN-α increases NK cell cytotoxicity by increasing perforin and granzyme release, and promotes NK proliferation through inducing interleukin (IL)-15 production by cells such as DCs and monocytes (Nguyen *et al.*, 2002). Type I IFNs can also induce the maturation of DCs *in vivo* and *in vitro* (Ito *et al.*, 2001; Montoya *et al.*, 2002). It is also known that type I IFNs can drive a potent T helper (Th)1 response (Cella *et al.*, 2000; Le Bon *et al.*, 2003).

The type I IFN response during acute HCV infection in humans has not been characterised. However, in the chimpanzee model, HCV infection is known to be rapidly followed by up-regulation of type I IFN-responsive genes in the liver (Bigger *et al.*, 2001; Su *et al.*, 2002). This appears to occur irrespective of the outcome of infection (Bigger *et al.*, 2001; Su *et al.*, 2002; Thimme *et al.*, 2002). It is likely that type I IFNs play an important role in controlling HCV infection, as HCV has several mechanisms for evading IFN effector pathways (discussed in detail later). However, although type I IFNs may limit viral spread in the early phase of infection, HCV nonetheless proceeds to establish a chronic infection in many infected individuals. During the chronic phase of infection, the production of type I IFN is clearly suboptimal as IFN therapy improves the control of virus replication in some patients with chronic HCV.

1.5.1.2. NK cells

NK cells play an important effector role in controlling many viral infections, especially in the early phase, and also have immunoregulatory functions (Bancroft *et al.*, 1981; Biron *et al.*, 1989; Brown *et al.*, 2001; Farrell *et al.*, 1997; French & Yokoyama, 2003;

Loh *et al.*, 2005; Shellam *et al.*, 1981; Yokoyama & Scalzo, 2002). The main mechanisms by which NK cells contribute to antiviral defence are via the lysis of virally-infected cells and the production of chemokines and cytokines such as IFN-γ. NK cells are activated by cytokines including IL-12, IL-15 and type I IFNs (Nguyen *et al.*, 2002). Triggering of NK cell effector functions is controlled by the balance of signalling through activating and inhibiting NK cell receptors (Lanier, 1998; Lanier, 2003; Lanier, 2005; Moretta & Moretta, 2004).

In humans, NK cells are typically defined as $CD56^{+}$ $CD3^{-}$ lymphocytes, and constitute 10-15 % of cells in peripheral blood. NK cells can be divided into $CD56^{dim}$ and $CD56^{high}$ NK subsets according to the density of expression of CD56 on the cell surface (Cooper *et al.*, 2001b; Jacobs *et al.*, 2001). $CD56^{dim}$ NK cells comprise 90 % of the NK population, and express CD16 (FcgRIII), which mediates antibody-dependent cellular cytotoxicity (ADCC), and high levels of perforin, enabling $CD56^{dim}$ NK cells to act as the main mediators of NK cytotoxicity (Cooper *et al.*, 2001a; Jacobs *et al.*, 2001). $CD56^{high}$ NK cells are a more minor NK population. They express low or no CD16, but express high levels of homing molecules that mediate cell migration to secondary lymph nodes (Frey *et al.*, 1998). Although $CD56^{high}$ NK cells exhibit weak cytotoxic activity, they are superior in producing cytokines, in particular IFN-γ (Cooper *et al.*, 2001b; Jacobs *et al.*, 2001). This NK cell subset is thus hypothesised to have an immunoregulatory role.

The importance of NK cell function in HCV infection is supported by the observation that individuals homozygous for human leukocyte antigen (HLA)-C1 and killer cell immunoglobulin-like receptor (KIR)2DL3 resolve HCV infection more frequently than individuals with other genotypes (Khakoo *et al.*, 2004; Parham, 2004). Several studies have reported that the cytolytic ability of NK cells is significantly decreased in

individuals chronically infected with HCV (Corado *et al.*, 1997; Gabrielli *et al.*, 1995). This may correlate with the fact that patients with chronic HCV infection have a reduced frequency of NK cells in the peripheral blood, and have altered proportions of $CD56^{dim}$ and $CD56^{high}$ subsets (Meier *et al.*, 2005). Notably, the frequency of NK cells can be normalised by IFN treatment (Van Thiel *et al.*, 1994).

1.5.1.3. NKT/NT cells

NKT and natural T (NT) cells are a heterogeneous population of lymphocytes expressing TCRs as well as surface markers characteristic of NK cells. NKT cells can be defined as a population of cells that are CD1d-restricted. They may express either an invariant TCR comprising the Vα24/JαQ and Vβ11, or a diverse repertoire of TCRs (Van Dommelen & Degli-Esposti, 2004). Functional and biochemical studies suggest that glycolipids are presented by CD1d expressed on APCs and liver epithelial cells (Park *et al.*, 1998). NKT cells can respond very rapidly to stimuli, exert cytotoxic activity and release large amounts of IFN-γ and IL-4 (Exley *et al.*, 2002; Kawano *et al.*, 1997; Metelitsa *et al.*, 2001). The importance of NKT cells in viral infections has been suggested by several reports (Ashkar & Rosenthal, 2003; Exley *et al.*, 2003; Grubor-Bauk *et al.*, 2003; Johnson *et al.*, 2002; Unutmaz, 2003). However, the contribution made by these cells to control of HCV infection has not been defined.

The liver generally contains a large numbers of NT cells, however, these cells are decreased in numbers in HCV infected patients (Deignan *et al.*, 2002; Kawarabayashi *et al.*, 2000). In addition, very few NKT cells expressing invariant TCRs are found in the livers from HCV^{+} subjects (Deignan *et al.*, 2002; Exley *et al.*, 2002).

1.5.1.4. DCs

DCs act as a bridge between innate and acquired immune responses (Banchereau *et al.*, 2000). DCs perform an effector role in the innate response (e.g. via production of type I IFNs) and can enhance the activation of other components of the innate immune response, for example DCs can activate NK cells via production of cytokines such as IL-12 and IL-15. DCs also play a key role in the induction and regulation of T cell responses, mediating antigen presentation, T cell co-stimulation and helping to direct the Th1 or Th2 bias of the response. There are different subtypes of DCs, including myeloid and plasmacytoid DCs in human peripheral blood. Myeloid DCs are suggested to be the progeny of $CD14^+$ monocytes (Dzionek *et al.*, 2000). They mediate antigen capture and processing, and after receipt of appropriate maturation stimuli, they can act as highly potent APCs (Dzionek *et al.*, 2000). As mentioned above, plasmacytoid DCs are the major type I IFN producers and play an important role in the Th1 polarisation of immune responses during viral infections (Cella *et al.*, 2000; Kadowaki *et al.*, 2000).

Despite conflicting data, a number of studies suggest that the functions of DCs may be impaired during HCV infection. A reduced frequency of myeloid and/or plasmacytoid DCs has been found in the periphery of chronic HCV patients by some (Anthony *et al.*, 2004; Kanto *et al.*, 2004) but not all groups (Piccioli *et al.*, 2005). Chronic HCV infection has also been shown to adversely affect the production of IL-12, IFN-γ and IFN-α by DCs (Anthony *et al.*, 2004; Kanto *et al.*, 1999; Kanto *et al.*, 2004) and the allostimulatory function of DCs (Auffermann-Gretzinger *et al.*, 2001; Bain *et al.*, 2001; Kanto *et al.*, 1999; Kanto *et al.*, 2004). Moreover, DCs from HCV infected patients have been reported to exhibit impairments in maturation in response to stimuli, maintaining an immature phenotype as characterised by surface marker expression and continued capacity to uptake antigen (Auffermann-Gretzinger *et al.*, 2001; Kanto *et al.*,

1999). Again, not all groups have observed this (Longman *et al.*, 2004; Piccioli *et al.*, 2005; Rollier *et al.*, 2003). It is unclear at what stage of infection these abnormalities in DC numbers/functions may be induced and what role they play in the establishment of viral persistence. However, defects in DC functions in chronically infected individuals may contribute to maintenance of HCV persistence through secondary effects on other cells of the innate immune system and/or on the HCV-specific cell response.

Fernandez *et al.* first proposed the importance of cross-talk between NK and DCs in the regulation of both innate and adaptive responses (Fernandez *et al.*, 1999). Others have subsequently supported the idea that NK cells and DCs reciprocally activate one-another during an immune response, and require both cell-cell contact and soluble factors for optimal co-stimulatory effects (Della Chiesa *et al.*, 2005; Ferlazzo *et al.*, 2002; Fernandez *et al.*, 1999; Gerosa *et al.*, 2002; Piccioli *et al.*, 2002). Interestingly, DCs from HCV infected patients were found to show defects in the production of IL-15 in response to IFN-α, which resulted in impairment of the induction of major histocompatibility complex (MHC) class I-related chain A or B (MICA/B) on the surface of DCs, and subsequently impairment of the activation of NK cells due to the lack of proper cell-cell contact (Jinushi *et al.*, 2003a; Jinushi *et al.*, 2003b). Serum IL-15 levels are reduced in patients chronically infected with HCV (Meier *et al.*, 2005). As IL-15 is required for the proliferation, survival and effector functions of NK cells (Carson *et al.*, 1995; Carson *et al.*, 1997; Carson *et al.*, 1994; Cooper *et al.*, 2002) and NT cells (Dunne *et al.*, 2001), and for the survival of $CD8^+$ T cells (Berard *et al.*, 2003), defects in IL-15 synthesis may contribute to the development and/or maintenance of persistent HCV infection.

1.5.2. Humoral immune responses

After acute infection with most viruses, Abs are produced by B cells that limit the spread of infection and also contribute to clearance of the infection. Abs act by blocking the entry of viruses into cells or neutralising their infectivity at post-entry steps; by opsonising free viral particles for uptake by phagocytes; and by targeting viral particles and infected cells for destruction through complement-mediated lysis or ADCC. Moreover, once infection has been resolved, virus-specific Abs remain in body for years, and play an important role in preventing re-infection on later exposure. The majority of vaccines in use today are thought to work primarily through induction of Ab responses.

In HCV infection, seroconversion normally occurs between 7-12 weeks post-infection (Farci *et al.*, 2000; Thimme *et al.*, 2001), which is late compared with many other viral infections. A qualitative and quantitative analysis of the humoral response to HCV in the acute phase of infection in patients who resolved or progressed to chronic infection showed that the production of Abs directed against different HCV antigens was limited, these Abs were largely restricted to the IgG1 isotype and the response was of relatively low titre (Chen *et al.*, 1999). There was no correlation between the outcome of infection and the nature of the early Ab response. On the other hand, Zibert *et al.* found that early Ab responses to HVR-1 of E2 were associated with clearance of HCV (Zibert *et al.*, 1997).

Analysis of the neutralising ability of Abs raised to HCV has been hampered by the inability to grow the virus *in vitro*. Neutralisation of the binding of E2 to CD81 expressed on Molt-4 cells (NOB assay) is frequently used as a surrogate for neutralising activity (Pileri *et al.*, 1998; Rosa *et al.*, 1996), but it is not clear how NOB activity

relates to the neutralisation of viral infectivity *in vivo*. More recently, retroviral particles pseudotyped with HCV glycoproteins have been used to determine the neutralising activity of Abs/sera (Bartosch *et al.*, 2003a; Keck *et al.*, 2004; Lavillette *et al.*, 2005a; Op De Beeck *et al.*, 2004). The neutralising ability of Abs has also been tested using the chimpanzee model (Farci *et al.*, 1994; Farci *et al.*, 1996).

The regions of HCV that exhibit the highest sequence variability are HVR-1 and HVR-2 within E2. The majority of neutralising Abs that block HCV infection are directed against HVR-1 (Zibert *et al.*, 1995). It has been reported that protection against HCV infection can be mediated by neutralising Abs directed against HVR-1 (Esumi *et al.*, 1999; Farci *et al.*, 1994; Farci *et al.*, 1996; Shimizu *et al.*, 1996). Further evidence in support of the role played by Ab responses against E2 HVR-1 in control of HCV replication comes from the observation that vigorous Ab responses against HVR-1 enhance the appearance of viral variants bearing mutations that confer escape from recognition by existing Abs (Kato *et al.*, 1994; Shimizu *et al.*, 1994) (Farci *et al.*, 2000; Ray *et al.*, 1999; Ray *et al.*, 2000; van Doorn *et al.*, 1995). In addition, some studies have reported the presence of neutralising Abs that are partially independent from HVR-1 and have a broad reactivity with different HCV genotypes (Habersetzer *et al.*, 1998; Hadlock *et al.*, 2000; Keck *et al.*, 2004; Lavillette *et al.*, 2005a; Meunier *et al.*, 2005; Roccasecca *et al.*, 2001; Rosa *et al.*, 1996; Steinmann *et al.*, 2004). The presence of strong humoral responses against multiple immunodominant epitopes is also correlated with lower viral load *in vivo* (Carlos *et al.*, 2004). These reports suggest that the humoral immune response makes a valuable contribution to the control of viral replication in infected individuals.

Nonetheless, it has been shown that viral clearance can occur in the absence of Ab responses to E1E2 in a chimpanzee model (Bassett *et al.*, 1999; Meunier *et al.*, 2005). Further, it has been reported that chimpanzees who had successfully recovered from acute HCV infection could be re-infected with homologous or heterologous HCV strains, with viraemia occurring despite the presence of anti-HCV Abs, implying failure of these Abs to protect against the infection (Farci *et al.*, 1992; Prince *et al.*, 1992; Wyatt *et al.*, 1998). Indeed, HCV-infected patients can develop virus-specific Abs against both structural and NS proteins, and still maintain viraemia.

It has been suggested that the effectiveness of neutralising Abs may be limited because of the ability of the evolving viral quasispecies to outpace the immune response. Co-existence of two types of quasispecies has been postulated: one that escapes from neutralising Abs and successfully persists, and another that has considerably divergent sequences, which may be preferentially neutralised (Abbate *et al.*, 2003; Hu *et al.*, 2005; Korenaga *et al.*, 2001; Ray *et al.*, 1999). The composition of the quasispecies shifts significantly during seroconversion, when disappearance of some of the most diverse viral variants has been observed to occur following the development of Abs, coinciding with a decrease in the viral load (Hu *et al.*, 2005; Lavillette *et al.*, 2005b). Interestingly, Hu *et al.* have reported that HCV escape mutants in HVR-1 of E2 are positively selected to mimic human immunoglobulin both antigenically and structurally (Hu *et al.*, 2005). The direct relationship between quasispecies distribution, viral complexity within HVR-1 during the acute phase of infection and persistence is consistent with a model where the more mutations E2 undergoes, the greater the probability that it will mimic host immunoglobulin and be able to evade recognition (Arenas *et al.*, 2004; Farci *et al.*, 2000; Hu *et al.*, 2005; Ray *et al.*, 2000).

Thus the humoral immune response does seem to play a protective role in HCV infection, although the virus may be able to evade this to establish a persistent infection. The outcome of infection is thought to be dependent on the nature of virus at initial infection, and the balance between host immune responses and viral evolution. Klenerman *et al.* also suggest that an appropriate balance between humoral and cellular immune responses is necessary for the clearance and effective control of HCV infection (Klenerman *et al.*, 2000).

1.5.3. Cell-mediated immune responses

Cellular immune responses play a key role in the elimination of many viral infections, because of their ability to recognise infected cells and destroy them or eliminate viral infection from them.

$CD8^+$ T cells recognise specific peptides presented on MHC class I molecules. The main functions of $CD8^+$ T cells are to lyse infected cells in an antigen-specific manner and produce cytokines such as IFN-γ and tumour necrosis factor (TNF)-α that inhibit viral replication without killing the infected cells. $CD4^+$ T helper cells recognise viral peptides in the context of MHC class II molecules. They mediate their activities by cognate interactions with other cell types and production of cytokines that have direct effector and immunomodulatory roles. $CD4^+$ T cells provide help for Ab responses by direct interaction with B cells and via production of Th2 cytokines (e.g. IL-4, IL-10) which activate B cells and induce isotype switching of Abs. $CD4^+$ T cells also provide help for $CD8^+$ T cells by mediating activation of professional APCs (through both cognate interactions and production of Th1 cytokines e.g. IFN-γ), which in turn activate and expand naïve $CD8^+$ T cells. $CD4^+$ T cells play an important role in the generation and maintenance of $CD8^+$ T cell memory (Bourgeois *et al.*, 2002; Janssen *et al.*, 2003;

Janssen *et al.*, 2005). In the absence of $CD4^+$ T help, $CD8^+$ T cells are likely to become exhausted and non-functional (Fuller *et al.*, 2004; Kalams & Walker, 1998; Zajac *et al.*, 1998). With the dependence of efficient $CD8^+$ CTL activity and humoral responses on $CD4^+$ T cells, deficiencies in $CD4^+$ T cell help can lead to poor control of viral replication by CTL and generation of Abs of restricted specificities (Klenerman *et al.*, 2000).

In HCV infection, it is suggested that vigorous multi-specific Th1 type $CD4^+$ T cell responses and CTL responses are important in both clearance of acute HCV infection and long-term control of chronic viraemia (Cramp *et al.*, 1999; Diepolder *et al.*, 1995; Hiroishi *et al.*, 1997; Lechner *et al.*, 2000a; Missale *et al.*, 1996). Thus the intensity, epitope specificity and cytokine profile of the T cell response during the early stages of HCV infection appear to determine the outcome of HCV infection. Efficient viral control by cellular immune responses before the significant emergence of Abs may lead to low selection pressure by Abs, and subsequently successful HCV clearance (Klenerman *et al.*, 2000). Patients that successfully recover from HCV infection maintain circulating HCV specific Th cells and CTL for many years even after the humoral immune response declines (Shoukry *et al.*, 2003; Takaki *et al.*, 2000). Conversely, $CD4^+$ and $CD8^+$ T cell responses are not sustained in chronically-infected HCV patients (Gerlach *et al.*, 1999; Lechner *et al.*, 2000b).

The majority of HCV-infected patients develop $CD4^+$ T cell responses to viral proteins. Evidence supporting the importance of $CD4^+$ helper T cells in the control of infection includes observations that virus clearance is associated with long-term maintenance of proliferative $CD4^+$/Th1 T cell responses against HCV NS proteins, in particular the viral

NS3 protein, whereas virus-specific $CD4^+$ T cell responses are lost in chronic patients (Diepolder *et al.*, 1995; Gerlach *et al.*, 1999). Chronically infected patients show significant numbers of activated antigen-specific $CD4^+$ T cells, but they are functionally impaired in proliferation and IFN-γ secretion (Ulsenheimer *et al.*, 2003). Treatment of these individuals with IFN-α has been shown to induce recovery of some Th responses, leading to a sustained response to therapy (Cramp *et al.*, 2000). In addition, there is a strong association between possession of HLA DQ1*0301/DRB1*1101 and spontaneous viral clearance (Thursz *et al.*, 1999). Patients with HLA DQ1*0301 show stronger HCV specific $CD4^+$ T cell responses than those without (Harcourt *et al.*, 2001).

In acute HCV infection, $CD8^+$ T cell responses against multiple viral proteins are observed in both the liver and blood (Koziel *et al.*, 1992; Koziel *et al.*, 1993; Koziel *et al.*, 1995). Studies in both humans and chimpanzees have shown that successful control of HCV replication is associated with vigorous, broad, persistent CTL responses during acute infection, and that HCV-specific CTL responses are generally much weaker in chronically infected patients (Cooper *et al.*, 1999; Gruner *et al.*, 2000; Hiroishi *et al.*, 1997; Lechner *et al.*, 2000a; Nelson *et al.*, 1997; Rehermann *et al.*, 1996). $CD8^+$ T cells from patients with both acute and chronic HCV infections have an "immature" stunned phenotype ($CD27^+$, $CD28^+$, $perforin^{low}$). At the peak of viraemia, HCV-specific $CD8^+$ T cells have been found to have an impaired ability to secrete IFN-γ and TNF-α, to proliferate after antigen stimulation and to exert cytotoxicity (Appay *et al.*, 2002; Gruener *et al.*, 2001; Lechner *et al.*, 2000a; Thimme *et al.*, 2001; Urbani *et al.*, 2002; Wedemeyer *et al.*, 2002). T cell dysfunction has been observed in all patients with acute HCV infection, irrespective of the virological outcome. Interestingly, the recovery of IFN-γ production by $CD8^+$ T cells coincides with the emergence of $CD4^+$ T cell

responses, a significant decrease in the viral load and self-limited infection (Thimme *et al.*, 2001; Urbani *et al.*, 2002). However, impairment of $CD8^+$ T cell effector functions persists when chronic infection develops (Urbani *et al.*, 2002). The impairment of $CD8^+$ effector functions seems to be restricted to HCV-specific T cells, because immunosuppression is not common in HCV infection and chronically-infected patients exhibit normal responses to other viruses (Lucas *et al.*, 2004; Wedemeyer *et al.*, 2002).

Natural regulatory T (T_{reg}) cells are also reported to play a role in HCV infection. T_{reg} cells are $CD4^+$ T cells that constitutively express CD25 and the T cell inhibitory receptor CTLA-4 and unique transcription factor Foxp3 (Belkaid & Rouse, 2005). These cells have an inhibitory effect on the Th1 response, but promote Th2 polarisation and protect the host from lethal inflammatory pathology. Patients chronically infected with HCV have more circulating natural T_{reg} cells in peripheral blood than uninfected people, and depletion of T_{reg} cells enhances antigen-specific $CD8^+$ T cell responses *in vitro* (Cabrera *et al.*, 2004; Sugimoto *et al.*, 2003).

Thus functional HCV-specific $CD4^+$ and $CD8^+$ T cells are required for successful clearance and control of HCV.

1.5.4. Role of the immune response in liver damage in patients chronically infected with HCV

The liver is enriched in $CD8^+$ T, NK and NT cells compared with the blood (Doherty *et al.*, 1999; Exley *et al.*, 2002). Upon viral infection in the liver, cells release type I IFNs, which in turn promotes infiltration and activation of NK cells (Salazar-Mather *et al.*, 1998). These act as an important source of IFN-γ and other cytokines and chemokines

(Fogler *et al.*, 1996), which play a major role in the recruitment and activation of virus-specific $CD4^+$ and $CD8^+$ T cells, leading to liver injury. Activated HCV-specific T cells have been reported to be present at 10-30-fold higher frequencies in the liver than in the blood (He *et al.*, 1999). Although $CD8^+$ T cell responses in patients with chronic HCV infection are too weak to mediate viral clearance, they may lead to liver damage and promote chronic inflammation in the liver. It has been reported that patients who exhibited intra-hepatic HCV-specific CTL activity had both lower levels of viraemia and more active liver disease histologically (Nelson *et al.*, 1997). Interestingly, HCV specific CTL can also kill uninfected hepatocytes *in vitro* (Ando *et al.*, 1997). This suggests that whilst the CTL response may be exerting some control over viral levels, it can also contribute to liver damage if it is not able to clear the infection from the liver.

1.6. Immune evasion strategies used by HCV

1.6.1. Overview of viral immune evasion strategies

In virus infections, there is a balance between the host immune response, which tries to eliminate the infection, and the virus, which tries to multiply and disseminate progeny to new hosts. To tip this balance in their favour, many viruses have evolved mechanisms to avoid control by the host immune response (Tortorella *et al.*, 2000). Viral immune evasion strategies can be divided into three categories: those involving (1) impairment of the immune response; (2) evasion of recognition by the host immune response; and (3) resistance to control by immune effector mechanisms. Given the high frequency with which it establishes persistence, HCV is likely to use a combination of strategies to combat the host immune response.

1.6.2. Impairment of the host immune response

Viruses may interfere with the induction or maintenance of the host immune response by inducing a generalised immunosuppression in the infected host. This may be achieved by different mechanisms. Firstly, viruses may encode proteins that suppress immune responses. For example, the envelop glycoproteins of a number of retroviruses (e.g. the p15E protein of feline leukemia virus and murine leukemia virus, the gp21 protein of human T-lymphotropic leukemia virus-1 and the gp41 protein of HIV-1) have been reported to contain immunosuppressive peptides that can inhibit T and B cell activation (Cianciolo *et al.*, 1985; Denner *et al.*, 1996; Ruegg *et al.*, 1989; Wang *et al.*, 1995). Secondly, immunosuppression by viruses often involves actual infection of immune cells, which may impair their functions or target them for destruction. HIV is one of the viruses that interferes with immune responses most spectacularly in this way. HIV infects $CD4^+$ T cells, monocytes, macrophages and DCs and impairs their functions or destroys them (Alimonti *et al.*, 2003; Chinen & Shearer, 2002; Mattapallil *et al.*, 2005). Measles virus also infects T cells, B cells, DCs and monocytes and impairs their functions or destroys them by formation of syncytia (Joseph *et al.*, 1975).

Alternatively, viruses may more selectively interfere with the induction/maintenance of the virus-specific immune response. If the host is infected during early embryonic life (following in utero LCMV infection in mice or HBV in humans), tolerance may be induced to viral antigens by the same mechanisms by which self-tolerance is achieved, leading to viral persistence. In adults, virus-specific responses may be impaired by "exhaustion" (deletion or functional impairment) of T cells (e.g. rapidly-replicating strains of LCMV cause exhaustion of virus-specific $CD8^+$ T cells, leading to a persistent infection (Doherty, 1993). The mechanisms involved are not fully understood, but may include "over-stimulation" of cells in the face of the high antigen loads and/or

alterations in APC functions.

As described in section 1.5, many arms of the immune response are impaired during HCV infection. In most cases, the precise mechanisms underlying the impairment are not understood.

It has been reported that DCs from HCV-infected patients have an impaired T cell stimulatory capacity. DCs are the most potent APCs and play an important role in the activation of T cell responses following viral infection. Defects in DC functions have been suggested to lead to abnormal priming and incomplete activation of HCV specific-$CD4^+$ T cells. Defects in APC functions and/or weak and defective $CD4^+$ T cell responses are likely to result in failure to induce and/or sustain strong, functional HCV-specific $CD8^+$ responses. In support of this, weak HCV-specific $CD4^+$ and $CD8^+$ T cell responses are seen in most chronically-infected patients (Cooper *et al.*, 1999; Gerlach *et al.*, 1999; Gruner *et al.*, 2000; Hiroishi *et al.*, 1997; Lechner *et al.*, 2000a; Nelson *et al.*, 1997; Rehermann *et al.*, 1996), and defects in the functional capacity of HCV-specific $CD8^+$ T cells have also been reported, as described in section 1.5.3.

The impaired functions of APCs and $CD8^+$ T cells may be partially explained by activities of the HCV core protein. Although there were variations in results, many studies found the expression of the HCV core protein can impair functions of DCs (Auffermann-Gretzinger *et al.*, 2001; Dolganiuc *et al.*, 2003; Hiasa *et al.*, 1998; Kim *et al.*, 2002; Lee *et al.*, 2001; Sarobe *et al.*, 2002), although this was not universally observed (Liu *et al.*, 2002). The core protein has also been shown to bind a complement receptor (C1qR), which can down-regulate production of IL-12 by macrophages and adversely affect proliferation and IL-2 and IFN-γ production by T cells (Eisen-Vandervelde *et al.*, 2004; Kittlesen *et al.*, 2000; Large *et al.*, 1999; Yao *et al.*, 2001; Yao

et al., 2004). In addition, it has been reported that the core protein can bind to the cytoplasmic tail of members of the TNF receptor family, including the lymphotoxin-β receptor, TNF receptor-1 and Fas. These are involved in triggering of apoptosis, and it is suggested that this interaction may sensitise cells to apoptosis (Chen *et al.*, 1997a; Hahn *et al.*, 2000; Zhu *et al.*, 2001).

There are also several reports that the HCV E2 protein can mediate immunomodulatory effects via binding to CD81; these are discussed below.

1.6.3. Evasion of recognition by the host immune response

One strategy that many viruses use to avoid recognition by the host immune system is infection of immunologically privileged sites. For example, HSV-1 and VSV can establish latent infections in central nervous system, where lymphocyte trafficking is limited due to the blood brain barrier and there is a low level of MHC class I expression. Evidence of HCV infection in the brain suggests that this may be a possible immune evasion strategy that HCV may employ (Radkowski *et al.*, 2002). More importantly however, the liver, where the majority of HCV replication occurs, has a number of unique immunological features that favour the induction of peripheral tolerance rather than induction of immunity to antigens expressing within it (likely because the liver is constantly expressed numerous antigens and toxic products). For example, activated $CD8^+$ T cells recruited to the liver frequently then undergo apoptosis (Mehal *et al.*, 2001). Antigen presentation to naïve $CD8^+$ T cells in the liver is incomplete, as APCs in the liver lack co-stimulatory molecules (Crispe, 2003). In addition, $CD4^+$ T cell responses in the liver are biased in a Th2 direction, which is partly due to IL-10 production by liver resident macrophage Kupffer cells (Knolle & Gerken, 2000). These features of the liver may contribute to the persistence of HCV.

Viruses may also avoid exposure to host immune responses by employing specialised mechanisms for cell-to-cell spread (e.g. syncytium formation by HIV and measles virus). Recently, it has been reported that in the presence of CD81 expression, some E1E2 proteins leave the ER, and associate with exosomes to be secreted into the extracellular space (Masciopinto *et al.*, 2004). Exosomes recovered from the plasma of patients with HCV infection contain HCV RNA, indicating the possibility that HCV may utilise the fusogenic property of exosomes to infect uninfected cells in the presence of HCV neutralising Abs (Masciopinto *et al.*, 2004).

As mentioned in section 1.4.2.4, HCV E1E2 can interact with DC-SIGN/DC-SIGNR. It is plausible that HCV may use DC-SIGN$^+$ cells (e.g. DCs) as carriers to facilitate the transport of virions to the liver in order to avoid the elimination of virus particles by immune clearance mechanisms before they reach the primary site of virus replication.

It is known that although HCV replication disrupts normal ER functions and induces ER stress, protein translation is elevated in cells with active HCV replication (Tardif *et al.*, 2002). The ER chaperone proteins Bip (GRP78) and GRP94 are involved in the ER stress response, activating an intracellular signalling pathway in response to the presence of misfolded proteins within the ER. Activation of Bip leads to further activation of proteins including the PKR-like ER-resident kinase PERK, which in turn inhibits protein synthesis via eIF2α (Tardif *et al.*, 2005). It is known that HCV E2 protein can transactivate the genes encoding Bip and GRP94 and bind tightly to Bip (Liberman *et al.*, 1999). As mentioned in section 1.4.1, aggregated E1 and E2 preferentially bind Bip, which supports a role of disulfide-linked aggregates of E1E2 *in vivo*. Although aggregated E1 and E2 induce ER stress, the E2 protein can bind to and

inhibit the activity of the ER stress-induced protein PERK through its PKR-eIF2α phosphorylation site homology domain (PePHD) (Pavio *et al.*, 2003). Thus it is plausible that HCV uses ER stress and manipulates cellular responses to ER stress to promote HCV persistence: the aggregates of E1E2 may play a role in the down-regulation of particle formation to minimize exposure of viral antigens to the immune system; and at the same time the E2 protein blocks ER stress-induced protein synthesis inhibition in order to maintain some level of production of virus proteins.

Another type of strategy many viruses employ to evade recognition by the host T cell response is interference with the presentation of antigens on infected cells, e.g. by inducing down-regulation of classical MHC molecule expression on the surface of infected cells. Viruses may target one or several parts of the process involved in generating MHC molecules expressing viral peptides, e.g. (1) inhibiting the transcription of MHC class I molecules (e.g. the Tat protein of HIV reduces MHC class I transcription (Jacobs *et al.*, 2005)); (2) interfering with the proteasomal proteolysis of proteins (e.g. the EBNA-1 protein from Epstein-Barr virus (EBV) is highly resistant to proteolysis (Levitskaya *et al.*, 1995)); (3) targeting the TAP transporter to block the transport of peptides (e.g. the ICP-47 protein from HSV blocks TAP transport from the cytosolic side (Fruh *et al.*, 1995)); (4) binding to and causing retention of newly synthesised class I molecules in the ER (e.g. the E3/19K protein from adenovirus retains MHC class I molecules in the ER (Andersson *et al.*, 1985; Burgert & Kvist, 1985)); (5) targeting MHC class I molecules for degradation by the proteasome (e.g. as mediated by the US2 and US11 proteins from human CMV (Schust *et al.*, 1998)); or (6) promoting internalisation of class I molecules from the cell surface for degradation by lysosomes

(e.g. the Nef protein of HIV reduces surface MHC class I levels in this way (Cohen *et al.*, 1999)).

Whether HCV uses this type of strategy to avoid detection and elimination by CTL is unclear. The expression of HCV proteins within cell lines *in vitro* has been reported not to affect cell-surface expression of MHC class I (Moradpour *et al.*, 2001). Herzer *et al.* found that the expression of the entire HCV genome or the core protein alone induced the up-regulation of MHC class I molecules on hepatic cell lines, due to induction of p53-dependent expression of TAP1 (Herzer *et al.*, 2003). In support of this, infections with other flaviviruses have been reported to be associated with the up-regulation of MHC class I molecules (Lobigs *et al.*, 1996). On the other hand, Tardif & Siddiqui found that cells expressing HCV subgenomic replicons had lower surface MHC class I molecule levels, due to interference with proper folding of these molecules (Tardif & Siddiqui, 2003). Although the precise mechanism is not known, it is speculated that ER stress induced by HCV replicons (Tardif *et al.*, 2002) may cause a reduction in protein glycosylation, which affects the folding and assembly of MHC class I molecules. In support of this, Tardiff & Siddiqui observed the accumulation of unfolded MHC class I molecules in replicon expressing cells (Tardif & Siddiqui, 2003).

Viruses may also use antigenic variation to escape from recognition by Abs or T cell responses. For example, CTL escape is thought to occur commonly in HIV-1 infection, and to play an important role in promoting viral persistence (Goulder & Watkins, 2004), and viral escape from neutralising Ab responses has also been documented in this infection (Wei *et al.*, 2003).

Antigenic variation leading to escape from both Ab and CTL responses has been shown to occur in HCV infection. Mutational changes have been found in particularly the

HVR-1 and HVR-2 regions within the HCV E2 protein in both humans and chimpanzees, some of which enable HCV to escape from Ab recognition (Farci *et al.*, 2000; Kato, 2001; Pavio & Lai, 2003; Ray *et al.*, 1999; Shimizu *et al.*, 1994). Acquisition of aa changes in or around CTL epitopes has also been described in chronic HCV infection in humans and chimpanzees (Chang *et al.*, 1997; Erickson *et al.*, 2001; Frasca *et al.*, 1999; Seifert *et al.*, 2004; Timm *et al.*, 2004; Tsai *et al.*, 1998; Weiner *et al.*, 1995). HCV escape has been reported to affect epitope processing (Seifert *et al.*, 2004; Timm *et al.*, 2004), MHC binding (Chang *et al.*, 1997) and TCR stimulation (Chang *et al.*, 1997; Erickson *et al.*, 2001; Tsai *et al.*, 1998). Viral escape can occur early during HCV infection (Timm *et al.*, 2004), but it is still not clear how important a contribution it makes to whether or not persistence is established.

Another mechanism that HCV employs to avoid immune responses is molecular mimicry, which is mediated through a region encompassing HVR-1 and HVR-2 of E2. Despite the high aa variability within HVR-1, the chemicophysical properties and conformation of HVR-1 are highly conserved (Penin *et al.*, 2001), and unrelated HVR-1 peptides show the extensive serological cross-reactivity in both acute and chronic HCV patients (Mondelli *et al.*, 2001). Interestingly, Hu *et al.* found that this region from different HCV genotypes is antigenically and structurally similar to human immunoglobulin variable domains (Hu *et al.*, 2005). It was also found that mutations in HVR are not random, but positively selected throughout the course of infection to increase similarity to immunoglobulin. Thus, HVR-1 is not only able to escape neutralising Abs but also to become non-immunogenic via molecular mimicry. Homology of E2 to immunoglobulin may also contribute to the autoimmune and lymphoproliferative diseases that HCV infection is associated with.

1.6.4. Evasion of control by immune effector mechanisms

Viruses may evade control by immune effector mechanisms by possessing resistance to antiviral cytokines and/or conferring resistance to lysis on infected cells. While RNA viruses tend to use antigenic variation as a major evasion strategy, mimicry of cytokines, chemokines and their receptors is more commonly used by large DNA viruses. For example, viruses may produce cytokine homologues to modulate cellular immune responses (e.g. an IL-10 homologue is produced by EBV (Alcami, 2003)) or soluble cytokine receptors to block the functions of antiviral cytokines (e.g. a viral TNF receptor is produced by cowpox virus and a viral IFN-γ receptor by vaccinia virus (Alcami, 2003)). Viruses may also interfere with the response of infected cells to cytokines, e.g. blocking IFN-induced signalling (e.g. the E3L protein from vaccinia virus inhibits PKR by interfering with the binding of PKR to double stranded RNA (Katze *et al.*, 2002)) and/or interfering with intracellular IFN-induced anti-viral effector mechanisms (e.g. the VH1 protein from vaccinia virus dephosphorylates signal transducer and activator of transcription (STAT)1 to block the IFN signalling cascade and the ICP0 protein from HSV-1 targets JAK-STAT signalling pathways of IFN (Katze *et al.*, 2002)). Some viruses confer resistance to lysis on infected cells (e.g. the Nef protein from HIV-1 inhibits Fas and TNF-α receptor-mediated apoptosis of infected cells by binding to and inhibiting apoptosis signal-regulating kinase-1 (Geleziunas *et al.*, 2001)).

Several HCV proteins have been suggested to confer resistance to the antiviral effects of type I IFNs, acting at multiple steps in the IFN induction/effector pathways (Capobianchi *et al.*, 2003; Hofmann *et al.*, 2005; Taylor *et al.*, 2000). These include the

NS3/NS4A protein, the core protein, the E2 glycoprotein and the NS5A protein.

The NS3/NS4A protein blocks the phosphorylation and nuclear translocation of interferon regulatory factor (IRF)-3, a critical transcription factor required for IFN gene induction (Foy *et al.*, 2003). More recently, it has been shown that the NS3/NS4A protein mediates cleavage of the adaptor protein Toll-IL-1 receptor domain-containing adaptor-inducing IFN-β (TRIF), which links Toll-like receptor (TLR)-3 to kinases responsible for activating IRF-3 and nuclear factor-κB (NF-κB) (Li *et al.*, 2005). In addition, the NS3 has been shown to bind to TANK-binding kinase-1 (TBK1) and inhibit its interaction with IRF-3 (Otsuka *et al.*, 2005). The NS3/4A protein has also been shown to disrupt type I IFN induction by the retinoic acid-inducible gene I (RIG-I) pathway, in this case acting downstream of IRF-3 and NF-kB activation (Breiman *et al.*, 2005; Foy *et al.*, 2005). HCV not only impairs type I IFN induction, but also its effector activities. The core protein induces degradation of STAT1, a protein involved in the IFN signalling pathway (Lin *et al.*, 2005).

The E2 protein can inhibit the IFN-inducible protein kinase PKR, which is activated during viral infection in response to the presence of double stranded RNA, and inactivate its anti-viral activity (Taylor *et al.*, 1999). Taylor *et al.* also found that a 12 aa sequence within E2, the PePHD (aa 659-670 in the genotype 1b HCV-J strain; (Kato *et al.*, 1990)) has homology to phosphorylation sites in PKR and eIF2α. The E2s from HCVs of genotype 1a and 1b have greater homology to the PKR and eIF2α phosphorylation sites than those of other HCV genotypes, which is speculated to enable better binding of genotype 1a and 1b E2s to these proteins (Taylor *et al.*, 1999). This may partly explain why infections with HCV genotype 1 are more resistant to IFN treatment than infections with other HCV genotypes (2a, 2b and 3a).

The NS5A protein is also able to bind PKR, through a part of its domain called the IFN

sensitivity determining region (ISDR). It blocks the homodimerisation of PKR, which is required for its anti-viral activity (Gale *et al.*, 1998; Gale *et al.*, 1997). It has been reported that the NS5A protein from HCV genotype 1b is more efficient in blocking the antiviral effects of IFN-α than that from HCV genotype 1a (Polyak *et al.*, 1999). It has also been shown that mutations in the ISDR result in loss of interaction of the NS5A protein with PKR (Gale *et al.*, 1998). In support of this, some studies showed that patients infected with HCV genotype 1b viruses that had more mutations within the ISDR exhibited a more sustained response to IFN therapy (Chayama *et al.*, 1997; Enomoto *et al.*, 1995; Gerotto *et al.*, 1999; Saiz *et al.*, 1998). However, this finding did not hold true in all geographical locations, and was not confirmed in other studies (Chung *et al.*, 1999; Khorsi *et al.*, 1997; McKechnie *et al.*, 2000; Squadrito *et al.*, 1997; Zeuzem *et al.*, 1997). Thus the relevance of mutations within the NS5A protein to PKR inhibition may be limited to particular genotype 1b HCV strains circulating in certain regions.

The NS5A protein may also inhibit the effects of type I IFNs through other mechanisms. Recently, expression of the NS5A protein in Hela cells was shown to lead to induction of IL-8, which counteracts the effect of IFN *in vitro* (Polyak *et al.*, 2001b). In support of this, lower levels of IL-8 were found in the serum of patients who showed a sustained response to IFN therapy (Mihm *et al.*, 2004; Polyak *et al.*, 2001a). In addition, the NS5A protein can block PKR-dependent signalling leading to impaired IRF-1 activation, which is involved together with IRF-3 in the regulation of antiviral gene expression (Pflugheber *et al.*, 2002).

Altogether, HCV thus appears to have evolved several mechanisms to circumvent the type I IFN response, the combined effects of which may contribute to the high rate of persistence of HCV and the resistance of this infection to IFN therapy.

The NS5A protein is also known to interact with many other cellular proteins to modulate signalling pathways (Macdonald & Harris, 2004). Further, it has been reported that the NS5A protein can block apoptosis by multiple mechanisms (Chung *et al.*, 2003; Lan *et al.*, 2002; Majumder *et al.*, 2002).

1.7. CD81

1.7.1. Biology of CD81

CD81 (formally known as TAPA-1) is a member of a large protein superfamily called the tetraspanins, which comprises at least 28 members in mammals (Hemler, 2001). Tetraspanins are evolutionarily conserved proteins expressed in diverse organisms including *Drosophila*, *Schistosoma* and *Caenorhabditis elegans* (Todres *et al.*, 2000). Within the family, CD81 and CD9 display close sequence homology (in humans, there is 23 % identity in the aa residues in their respective LEL domains), whilst the human CD82-LEL is the most distantly related to the human CD81-LEL (Kitadokoro *et al.*, 2001). Some tetraspanins have a broad distribution, whereas others have a very restricted pattern of expression.

All cells in the immune system express tetraspanins (Tarrant *et al.*, 2003). One of the unique features of tetraspanins is their ability to associate with each other and with many other membrane proteins to form a network called the tetraspanin-web. This helps to provide topological organisation on the cell surface by clustering appropriate molecules mainly in specialised cholesterol-rich membrane microdomains called lipid rafts. The interactions between tetraspanins and their associated proteins are highly specific and may in turn allow the modulation of intercellular immune interactions,

including cell motility, adhesion and homotypic cell aggregation, and enable signalling involving kinases to take place by clustering appropriate molecules in membrane-signalling complexes.

Specific interaction between CD81 and a wide range of membrane proteins has been reported (Boucheix & Rubinstein, 2001; Tarrant *et al.*, 2003). CD81 is reported to associate with CD19, CD21 and the IFN-inducible antigen Leu-13 on B cells (Fearon & Carter, 1995); CD3, CD4, CD8 and CD82 on T cells (Imai & Yoshie, 1993; Mannion *et al.*, 1996); HLA-DR on B cells and DCs (Engering & Pieters, 2001; Rubinstein *et al.*, 1996; Schick & Levy, 1993; Szollosi *et al.*, 1996; Vogt *et al.*, 2002); and CD14 on monocytes (Pfeiffer *et al.*, 2001; Underhill, 2003). CD81 is also found associated with several integrins: α3β1 on endothelial cells and α4β1 (VLA-4) on B and T cell lines, with the associations occurring through their common β1 integrin subunit (Mannion *et al.*, 1996; Yanez-Mo *et al.*, 1998).

In addition, it is reported that CD81 and CD9 form a very stable, specific and tight association with EWI-2 and EWI-F, which are widely expressed in many tissues including hepatocytes, B, T and NK cells (Charrin *et al.*, 2003; Stipp *et al.*, 2001). It has been shown that CD81 and CD9 physically link EWI-2 to α3β1 and α4β1 integrins (Kolesnikova *et al.*, 2004; Stipp *et al.*, 2003b).

Other than the membrane proteins with which it associates in cis, no natural ligand for CD81 has been identified. However, direct evidence for the existence of a natural CD81 ligand comes from a study on the role of CD81 as a mediator for neuronal signalling of astrocyte proliferative arrest (Kelic *et al.*, 2001). As discussed in section 1.4.2.1, the HCV E2 protein binds to CD81, an interaction that is thought to play a role in the HCV

attachment/entry process. HCV is not the only organism utilising CD81 as a receptor; *Plasmodium falciparum* and *Plasmodium yoelii* sporozoites also infect hepatic cells in a CD81-dependent manner (Silvie *et al.*, 2003).

1.7.2. Functions of CD81 on cells of the immune system

One of the important features of CD81 is its ability to associate with a wide range of membrane proteins, including homo/heterodimers of other tetraspanin superfamily proteins (Kitadokoro *et al.*, 2001). These interactions enable CD81 to generate different biological responses in different cell types. In general, the LEL contains the crucial functional sites mediating specific protein-protein interactions (Levy & Shoham, 2005; Stipp *et al.*, 2003a).

The association of CD81 with CD19, CD21 and Leu-13 on B cells plays a role in B cell activation (Fearon & Carter, 1995). Here, mAb ligation of CD81 has been shown to lower the threshold for B cell activation and proliferation induced by signal transduction through the B cell receptor (BCR) (Fearon & Carter, 1995). It is speculated that CD81 mediates this effect by bridging antigen-specific recognition and CD21-mediated complement recognition and prolonging the association of the BCR with signalling-active lipid rafts (Cherukuri *et al.*, 2004; Dykstra *et al.*, 2001; Levy & Shoham, 2005).

On T cells, CD81 associates with CD3, CD4, CD8 and CD82, providing a co-stimulatory signal to that induced by anti-CD3 Abs (Imai & Yoshie, 1993; Mannion *et al.*, 1996; Wack *et al.*, 2001). Several other molecules are also reported to co-stimulate CD3-mediated activation of T cells, including CD28, CD9, CD53 and CD82 (Lagaudriere-Gesbert *et al.*, 1997; Lebel-Binay *et al.*, 1995). A distinct feature of CD28-mediated co-stimulation is the sustenance of a high level of IL-2 production (Lenschow *et al.*, 1996). Interestingly, CD81-mediated co-stimulation resembles CD28-

mediated co-stimulation in human T cells, since CD81 can also lead to a sustained increase in IL-2 production (at a similar level to CD28) (Wack *et al.*, 2001). Conversely, in mouse T cells, CD81-mediated co-stimulation does not induce IL-2 production and is more similar to co-stimulation mediated via other tetraspanins (Tai *et al.*, 1997; Witherden *et al.*, 2000; Yashiro *et al.*, 1998). Another difference between CD81 on T cells in humans and mice is that the expression of CD81 on human T cells is down-regulated after cell activation (Fritzsching *et al.*, 2002), whilst CD81 expression levels increase following T cell activation in the mouse (Witherden *et al.*, 2000). In addition, CD81 cross-linking also results in activation of γδ T cells, both enhancing activation in response to CD3 signalling and stimulating IFN-γ production and enhanced IL-2-induced proliferation in the absence of anti-CD3 stimulation (Tseng *et al.*, 2001a; Tseng *et al.*, 2001b).

CD81 is also thought to be involved in the development of Th2 responses. During the development of Th2 responses, CD81 expression on B cells is required to induce antigen-specific IL-4 secretion by T cells and to produce optimal antibody responses to Th2 stimuli (Maecker *et al.*, 1998). However, Deng *et al.* reported that CD81 expression on T cells is also critical for cognate T-B cell interaction leading to Th2 responses (Deng *et al.*, 2002). Supporting a role for CD81 in T-B cell interaction, B cell activation enhances IL-4 and IL-10 production by T cells after CD81 ligation in human B and T cell co-culture systems (Secrist *et al.*, 1996). This effect is specific to T-B cell interaction, as when the B cells were replaced by monocytes, the same effect was not observed. Further, involvement of CD81 in T cell interaction with APC is supported by Mittelbrunn *et al.* who observed CD81 re-distribution to the contact area of T-B cell and T-DC conjugates in an antigen-dependent manner (Mittelbrunn *et al.*, 2002). It is not

known whether the presence of CD81 on DCs is required for the induction of antigen-specific Th2 responses by T cells.

It is also reported that ligation of CD81 induces an anti-proliferative effect in B cells (Oren *et al.*, 1990). Other functions of CD81 include the homotypic adhesion of cells. T and B cells are induced to aggregate by anti-CD81 mAbs (Levy *et al.*, 1998; Todd *et al.*, 1996).

CD81 also plays an important role in the tissue-specific trafficking of cells. CD81 regulates the adhesion of α4β1 (VLA-4) on U937 monocytes and B cells to vascular cell adhesion molecule (VCAM)-1 (Feigelson *et al.*, 2003). In addition, the presence of CD81 is obligatory to facilitate PMA-induced enhancement of integrin avidity (Feigelson *et al.*, 2003). In these systems, CD81 does not require interaction with a ligand or mAbs against the LEL.

CD81 has been shown to associate with certain intracellular signalling components in B and T cell lines; these include phosphatidylinositol 4-kinase (PI4K), which is an important enzyme in the synthesis of phosphatidylinositols, and protein kinase C (PKC) isozymes, which are known to have a role in integrin-mediated adhesion (Berditchevski *et al.*, 1997; Zhang *et al.*, 2001). Despite these reports, it is also suggested that CD81 may not act via itself transducing signals, but may exert its effects by promoting signalling through molecules with which associates, such as integrins.

The functions of CD81 in the immune system have also been evidenced by the abnormalities in immunity observed in CD81 knock-out (KO, $CD81^{-/-}$) mice. Study of $CD81^{-/-}$ mice illustrated the importance of CD81 complexes on B cells; $CD81^{-/-}$ mice

show reduced expression of CD19 on their B cells, and exhibit impaired CD19 signalling, as well as a reduction in B-1 cell numbers (Maecker & Levy, 1997; Miyazaki *et al.*, 1997; Tsitsikov *et al.*, 1997). The dependence of CD19 expression on CD81 expression is specific, as B cells from CD9 KO mice have normal levels of CD19 (Shoham *et al.*, 2003). It has been found that $CD81^{-/-}$ mice fail to develop strong Ab responses to type II thymus-independent antigens e.g. trinitrophenol-Ficoll and to antigens delivered in adjuvants that preferentially stimulate Th2 responses (e.g. ovalbumin given in alum) (Maecker *et al.*, 1998). They also show reduced allergen-induced hyper-reactivity (Deng *et al.*, 2000), which is thought to be due to defects in Th2 cytokine production, again supporting a role for CD81 in Th2 responses.

Although CD81 is expressed on multiple haematopoietic cell types in addition to B and T cells, its role in their functions is not well understood. At the time this project was started, nothing was known about the functions of CD81 on NK cells; this was thus one of the questions I addressed this in thesis. Very little was also known about role of CD81 on monocytes, macrophages and DCs; I thus also investigated possible role(s) of CD81 on DCs in this thesis.

1.7.3. Immunomodulatory effects of CD81 cross-linking by HCV E2

The multiple functions of CD81 on immune cells suggest that E2 binding to CD81 may have an immunomodulatory effect during HCV infection. In support of this, cross-linking of CD81 on T cells, and subsequently also B cells and NK cells by E2 has been reported to affect their activation and functions (Cocquerel *et al.*, 2003; Crotta *et al.*, 2002; Flint *et al.*, 1999b; Rosa, 2001; Tseng & Klimpel, 2002; Wack *et al.*, 2001). Strikingly, CD81 cross-linking by E2 was found to mediate very different effects on T

cells, B cells and NK cells, in line with the differing effects of CD81 ligation by Abs on these cell types.

Cross-linking of CD81 on T cells by E2 was found to lower the activation threshold for TCR-mediated proliferation and to induce a co-stimulatory signal that enhanced the secretion of IFN-γ, IL-4 and regulated on activation normal T-cell-expressed and secreted (RANTES), plus enhanced TCR and CCR-5 down-regulation (Nattermann *et al.*, 2004; Wack *et al.*, 2001). CD81-mediated co-stimulation triggered by HCV E2 may allow priming of T cell clones that receive sub-optimal stimuli and enhance the clonal expansion of T cells in lymph nodes. It may also increase the number of activated T cells infiltrating into the liver, which in turn may cause inflammation of the liver and the development of liver diseases in HCV infection.

Engagement of CD81 expressing-B cell lines by soluble E2 or E1E2 heterodimers resulted in cell aggregation (Cocquerel *et al.*, 2003; Flint *et al.*, 1999b) and protein tyrosine phosphorylation, indicative of B cell activation (Cocquerel *et al.*, 2003). Rosa *et al.* also found that the binding of recombinant E2 protein to CD81 on B cells delivered a very modest co-stimulation signal that could promote the full activation of B cells, demonstrated by cell proliferation and Ab production (Rosa, 2001). This fits well with the observations that CD81 is required for membrane re-organisation on B cells that are responding to co-engagement of the BCR and CD21 (Cherukuri *et al.*, 2004), and mAb ligation of CD81 lowers the threshold for B cell activation induced by signal transduction through the BCR (Fearon & Carter, 1995).

If E2-CD81 interaction promotes the expansion and proliferation of B cells, this may contribute to the development of some of the autoimmune diseases and B cell lymphomas associated with chronic HCV infection. In support of this, it was found that the interaction of E2 with CD81 induces hypermutation of the immunoglobulin gene in B cells and TNF-α production (Machida *et al.*, 2005).

Notably, insertion and deletion of aa within HVR-1 has been reported to be associated with mixed cryoglobulinemia type II, indicating that the chemicophysical properties of HVR1 may affect the E2 binding to B cells, which in turn may result in B cell proliferation (Gerotto *et al.*, 2001). However, although B cell clonal expansion is observed in the liver from the individuals with chronic HCV infection and cryoglobulinemia (Dammacco *et al.*, 1998; Sansonno *et al.*, 1998), the accumulated B cells in blood are naïve, resting cells, and have not undergone antigen-mediated activation and proliferation (Ni *et al.*, 2003). Thus the mechanism by which HCV infection causes autoimmune diseases is still not clear.

During my PhD, it was also reported that cross-linking of CD81 on NK cells by E2 results in the inhibition of NK cell cytotoxicity, proliferation and production of cytokines (IFN-γ and TNF-α) (Crotta *et al.*, 2002; Tseng & Klimpel, 2002). The inhibitory signal after CD81 ligation was found to be independent of the well-described negative signalling pathways in NK cells.

E2-CD81 interaction may thus play important roles in both the establishment of persistent infection by HCV and the pathogenesis of diseases associated with chronic HCV infection: HCV may evade early immune responses by inhibiting NK cell responses; HCV-activated autoreactive T cells may contribute to liver damage and

autoimmune disorders; and interaction of HCV with B cells may also contribute to autoimmune phenomena.

1.8. Objectives

Further analysis of the roles of CD81 in the immune response and the effects of E2 binding to CD81 on the functions of different subsets of immune cells are likely to give more insight into both HCV persistence and development of various HCV-related diseases.

The goal of this project was to understand the interaction of the HCV E2 protein with CD81 and other putative receptors on PBMC subsets, and the potential for CD81 cross-linking to modulate innate responses. Different forms of E2 proteins were used to investigate the interaction of the E2 protein with different PBMC subsets. To gain insight into the potential for E2-CD81 interaction to modulate the host innate response, the effect of antibody-mediated cross-linking of CD81 on NK cell and DC responses was investigated. In addition, functions of CD81 on DCs were examined using cells from $CD81^{-/-}$ mice.

In the long term, a better understanding of immunomodulatory effects of HCV E2 interaction with CD81 may clarify mechanisms involved in HCV persistence and HCV-related pathogenesis, and potentially facilitate the development of a prophylactic vaccine (lacking detrimental immunomodulatory effects) and new therapeutic strategies which act by blocking or reversing detrimental consequences of E2-CD81 interaction.

Chapter 2 Materials and Methods

2.1. Materials

2.1.1. Plasticware and other laboratory supplies

Product	Catalogue No.	Supplier
13 mm Coverslips	ML-579-13	Western Lab Suppliers, Aldershot, UK
12-well Flat bottom Plates	3513	Corning Costar, High Wycombe, UK
140 mm Nunclon TM Dishes	168381A	Life Technologies, Paisley, UK
15 ml Falcon Tubes	352096	Becton Dickinson Labwear, UJ, USA
48-well Plates	150687	Life Technologies, Paisley, UK
50 ml Falcon Tubes	352070	Becton Dickinson Labwear, UJ, USA
6-well Plates	353046	Becton Dickinson Labwear, UJ, USA
96-well V bottom Plates	3894	Corning Costar, High Wycombe, UK
96-well Flat bottom microplates (plate-bound system)	655161	Greiner Bio-One Ltd., Stonehouse, UK
96-well microtitre plate (Luciferase assay)	3917	Corning Costar, High Wycombe, UK
96-well Sample Plate (chromium release assay)	1450-401	Wallac, Turku, Finland
Bijou Tubes	275/0460/24	BDH Lab Supplies, Poole, UK
Cell Strainers 40 µm	352340	Becton Dickinson Labwear, UJ, USA
Centricon Plus-80	UFC5LGC08	Millipore Corp, Bedford, USA
Centriprep YM-10 10,000 MWCO	4304	Millipore Corp, Bedford, USA
Cluster Tubes (FACS tubes)	AB-0672	Abgene, Surrey, UK

Product	Catalogue No.	Supplier
Cuvettes Semi-micro 1.5 ml	6000-2111	The LabSales Company, Over, UK
Eppendorf Tubes 0.5 ml	96.4625.9.01	Tref Ag, Degersheim, Switzerland
Eppendorf Tubes 1.5 ml	96.8160.9.02	Tref Ag, Degersheim, Switzerland
FACS Tubes 5 ml	352054	Becton Dickinson Labwear, UJ, USA
Finntips	9401-255	Life Sciences, Basingstoke, UK
Flasks T150	90151	Helena Biosciences, Sunderland, UK
Flasks T25	9026	Helena Biosciences, Sunderland, UK
Flasks T75	9076	Helena Biosciences, Sunderland, UK
Haemacytometer		Weber Scientific International, Lancing, UK
High Performance Chemiluminescence Film (5x7)	RPN1674K	Amersham Biosciences, Little Chalfont, UK
High Performance Chemiluminescence Film (8x10)	RPN2114K	Amersham Biosciences, Little Chalfont, UK
HiTrapProtein G HP	17-0404-03	Amersham Biosciences, Little Chalfont, UK
Hybond ECL nitrocellulose membrane	RPN303D	Amersham Biosciences, Little Chalfont, UK
Immulon-II ELISA Plates	3455	Dynex technologies, Middlesex, UK
Immuno 96-well Nunc ELISA Plates (Maxisorp)	439454A	Life Technologies, Paisley, UK
Internal Thread 1.8 ml Nunc Cryovials	377267	Life Technologies, Paisley, UK
LS+ MACS Columns	130-042-401	Miltenyi Biotech, Bisely, UK
Microcon YM10	42406	Millipore Corp, Bedford, USA
Microscope Slides	406-0184-04	BDH Lab Supplies, Poole, UK
MS^+ MACS Columns	130-042-201	Miltenyi Biotech, Bisely, UK

Product	Catalogue No.	Supplier
Needle, 19G 1.1 x 40 mm	197	Terumo Corp,
Needle, 27G,	Z192384-100EA	Sigma Aldrich, Poole, UK
Pasteur Pipettes	475-068	Jencons Scientific, Leighton Buzzard, UK
PCR Reaction Tubes	2441/3004/10	Merck BDH Ltd., Lutterworth, UK
Petri dish, 100x150 mm style (BMDC dish)	35-1029	Becton Dickinson labwear, UJ, USA
Pipettes 10 ml	47110	Bibby Sterilin, Stone, UK
Pipettes 25 ml	18327	Bibby Sterilin, Stone, UK
Pipettes 50 ml	357550	Becton Dickinson labwear, UJ, USA
Pipettes 5 ml	40105	Bibby Sterilin, Stone, UK
Plate Sealers	EC/005.SPN	Camlab Ltd, Cambridge, UK
Slide-A-Lyzer (1000 MWCO) Dialysis Cassettes	6638122	Pierce & Warriner UK Ltd., Chester, UK
Standard Repette Dispenser Tips, 2.5 ml	488-007	Jencons Scientific, Leighton Buzzard, UK
Sterile Repette Dispenser Tips, 5 ml	488-008	Jencons Scientific, Leighton Buzzard, UK
Syringes 2, 25, 50 ml		IAH Stores, Compton, UK
Tips 1000 ul	GPS1000	Rainin Instruments Co Ltd., Woburn, USA
Tips 200 ul	GPS250	Rainin Instruments Co Ltd., Woburn, USA
Tips 20 ul	GPS25	Rainin Instruments Co Ltd., Woburn, USA
TSK G4000PWXL	8022	Anachem Ltd, Luton, UK
Universal Tubes	275/0460/04	BDH Lab Supplies, Poole, UK

2.1.2. Chemicals and biological reagents

Product	Catalogue No.	Supplier
100 bp DNA Ladder	G210A	Promega, Southampton, UK
1kb DNA Ladder	G571A	Promega, Southampton, UK
250 bp DNA Ladder	10596-013	Invitrogen Life Technologies, Paisley, UK
30% Acrylamide/bisacrylamide 29:1	161-0156	BioRad Laboratories Ltd, Hemel Hempstead, UK
Agarose LE Analytical Grade	A9539	Sigma Aldrich, Poole, UK
Ammonium Persulphate	161-0700	BioRad Laboratories Ltd, Hemel Hempstead, UK
Ampicillin		IAH Media Supplies, Compton, UK
B Cell Isolation Kit	130-046-901	Miltenyi Biotec, Bisley, UK
BD Cytofix/Cytoperm Kit	555028	BD biosciences PharMingen, Oxford, UK
BDCA-4 Cell Isolationi Kit	130-090-532	Miltenyi Biotec, Bisley, UK
Beta Plate Scint	SC/9200	Wallac, Turku, Finland
Blue/Orange 6x Loading Dye	G190A	Promega, Southampton, UK
Bovine Serum Albumin	A7030	Sigma Aldrich, Poole, UK
Calcium Phosphate Transfection Kit	CAPHOS	Sigma Aldrich, Poole, UK
CD11c (N418) MicroBeads (mouse)	130-052-001	Miltenyi Biotec, Bisley, UK
CD14 MicroBeads	130-050-201	Miltenyi Biotec, Bisley, UK
CD1c (BDCA-1) Dendritic Cell Isolation Kit	130-090-506	Miltenyi Biotec, Bisley, UK
CD3 MicroBeads	130-050-101	Miltenyi Biotec, Bisley, UK
Collagenase Type III	LS004182	Worthington Biochemical Corp. Reading, UK
Complete Protease Inhibitor Cocktail Tablets	1836-145	Boehringer Mannheim UK, Lewes, UK
Coomassie Brilliant Blue R250 Destain	161-0438	BioRad Laboratories Ltd, Hemel Hempstead, UK
Coomassie Brilliant Blue R250 Stain	161-0436	BioRad Laboratories Ltd, Hemel Hempstead, UK

Product	Catalogue No.	Supplier
Deoxynucleotide Triphosphates	10297-018	Invitrogen Life Technologies, Paisley, UK
Dimethyl sulphoxide (DMSO)	D2650	Sigma Aldrich, Poole, UK
DNAse I	AMPD1	Sigma Aldrich, Poole, UK
ECL Western Blotting Detection Regagents	RPN2108	Amersham Biosciences, Little Chalfont, UK
Ethanol		IAH Stores, Compton, UK
Ethidium Bromide	E1510	Sigma Aldrich, Poole, UK
Ethylenediaminetetraacetic acid (EDTA)	100935V	BDH Lab Supplies, Poole, UK
Formaldehyde	47629	Sigma Aldrich, Poole, UK
GeneJuice Transfection Reagent	70967-3	CN Biosciences, Beeston, UK
GenElute HP Plasmid Maxiprep Kit	NA0310	Sigma Aldrich, Poole, UK
Glycerol	101186M	BDH Lab Supplies, Poole, UK
Glycine	15709-017	Invitrogen Life Technologies, Paisley, UK
Heparin 1000 IU/ml ampoule		Leo Laboratories Ltd, Princes Risborough, UK
HisTrap Kit	17-1880-01	Amersham Biosciences, Little Chalfont, UK
Human IL-12	219-IL	R&D Systems, Abingdon, UK
Human IL-12 p40 ELISA Kit	DY1240	R&D Systems, Abingdon, UK
Human IL-12 p70 ELISA Kit	DY1270	R&D Systems, Abingdon, UK
Human IL-15	247-IL	R&D Systems, Abingdon, UK
Human IL-18	B001-5	MBL, Woburn, USA
Human IL-4	204-IL-025	R&D Systems, Abingdon, UK
Human IL-2	799068	Roche diagnostics Ltd., Lewes, UK
Human male AB serum	H4522	Sigma Aldrich, Poole, UK
Isopropanol	59080	Sigma Aldrich, Poole, UK
Kanamycin Sulfate	11815-024	GibcoBRL, Paisley, UK
Kpn I (Asp718)	15232-010	Invitrogen Life Technologies, Paisley, UK
Laemmli Sample Buffer	161-0737	BioRad Laboratories Ltd, Hemel Hempstead, UK
Lectin from *Galanthus nivalis* (GNA Lectin)	L8275	Sigma Aldrich, Poole, UK
Lipopolysaccharide (LPS) *E.coli* 055.135	L2880	Sigma Aldrich, Poole, UK
Luciferase Assay System	E1500	Promega, Southampton, UK

Product	Catalogue No.	Supplier
Luria Broth (LB)		IAH Media Supplies, Compton, UK
Luria Broth Agar Plates		IAH Media Supplies, Compton, UK
Marvel		Premier International Food Ltd, Spalding, UK
Methanol	10158BG	BDH Lab Supplies, Poole, UK
Micro Protein Determination	610-A	Sigma Diagnostics,
Molecular Weight Rainbow Marker	RPN800	Amersham Biosciences, Little Chalfont, UK
Monocyte Negative Isolation Kit	113.09	Dynal Biotech Ltd, Wirral, UK
Mouse CD11c (N418) MicroBeads	130-052-001	Miltenyi Biotec, Bisley, UK
Mouse IFN-α/ β	Gift from Dr Arun Khamas	Le Bon *et al*., 2001
Mouse IL-12 p70 ELISA kit	DY419	R&D Systems, Abingdon, UK
Mouse IL-18	PMC0184	Biosource, Nivelles, Belgium
Mouse IL-2	1271164	Roche diagnostics Ltd., Lewes, UK
NK Cell Isolation Kit, human	130-046-502	Miltenyi Biotec, Bisley, UK
NK Cell Isolation Kit, mouse	130-090-864	Miltenyi Biotec, Bisley, UK
Pan T Cell Isolation Kit	130-053-001	Miltenyi Biotec, Bisley, UK
Phosphate Buffer Saline x10	14200-067	GibcoBRL, Paisley, UK
Phytohemaglutinin (PHA) (Lectin from *Phaseolus vulgaris*)	L-9132	Sigma Aldrich, Poole, UK
QIAfilter Plasmid Maxi Kit	12263	Qiagen, Crawley, UK
Rabit anti-human Ig antibodies coupled with polyacrylamide beads	15378	Rochford medical Ltd., High Wycombe, UK
Recombinant Human GM-CSF	Gift from Dr John Tite	GlaxoSmithKline, Stevenage, UK
Recombinant Mouse GM-CSF	415-ML	R&D Systems, Abingdon, UK
Sodium acetate (anhydrous)	S2889	Sigma Aldrich, Poole, UK
Sodium Azide	S-8032	Sigma Aldrich, Poole, UK
Sodium Carbonate Anhydrous	102405Y	BDH Lab Supplies, Poole, UK
Sodium Chloride	102415K	BDH Lab Supplies, Poole, UK
Sodium Dodecyl Sulphate (SDS)	444464T	BDH Lab Supplies, Poole, UK
Sodium Hydrogen Carbonate	102475W	BDH Lab Supplies, Poole, UK
Subcloning Efficiency DH5α Competent Cells	18265-017	Invitrogen Life Technologies, Paisley, UK
Sucrose	102744B	BDH Lab Supplies, Poole, UK

Product	Catalogue No.	Supplier
Sulphuric Acid	102761C	BDH Lab Supplies, Poole, UK
Super Taq PCR Buffer 10x	TPO5X	HT biotech, Cambridge, UK
TAE 25x	0796-1.6L	Amresco, Ohio, USA
Taq DNA Polymerase	10342-020	Invitrogen Life Technologies, Paisley, UK
TEMED	161-0800	BioRad Laboratories Ltd, Hemel Hempstead, UK
Tetramethylbenzidine (TMB) Substrate Reagent Pack	DY999	R&D Systems, Abingdon, UK
Tris/Glycine SDS Buffer 10x	161-0732	BioRad Laboratories Ltd, Hemel Hempstead, UK
Tris-HCL	T3253	Sigma Aldrich, Poole, UK
Triton-X-100	T9284	Sigma Aldrich, Poole, UK
Trizma Base	T1503	Sigma Aldrich, Poole, UK
Tween-20	P7949	Sigma Aldrich, Poole, UK
Zenon Alexa Fluor 488 Mouse IgG1 Labeling Kit	Z-25002	Molecular Probes, Leiden, Holland

2.1.3. Cell lines

Cell Line	Catalogue No.	Supplier
293T		Prof. Mario Stevenson (University of Massachusetts, Worcester, USA)
Hep16		Dr Arvind Patel (MRC Virology Unit, Glasgow, UK)
Hep3B	86062703	European Collection of Cell Cultures
Huh-7		Dr Arvind Patel (MRC Virology Unit, Glasgow, UK)
K562	89121407	European Collection of Cell Cultures
293FT		Dr Tim Hickling (Division of Microbiology and infectious diseases, University of Nottingham, UK)
Molt-4		Dr Persephone Borrow, (The Edward Jenner Institute for Vaccine Research, Comnpton, UK)
HepG2		Dr Arvind Patel (MRC Virology Unit, Glasgow, UK)

2.1.4. Tissue culture reagents

Product	Catalogue No.	Supplier
Accutase	L11-007	PAA laboratories, Linz, Austria
DMEM with Glutamax Medium	31955-021	GibcoBRL, Paisley, UK
FACS Lysing Solution 10x	349202	Becton Dickinson Labwear, UJ, USA
Foetal Calf Serum (FCS)	A15-002	PAA laboratories, Linz, Austria
Geneticin, liquid	10131-019	Invitrogen, Paisley, UK
Good Foetal Calf Serum (GFCS)	10106-169	Invitrogen, Paisley, UK
Histopaque-1077	1077-1	Sigma Aldrich, Poole, UK
Histopaque-1083	1083-1	Sigma Aldrich, Poole, UK
Low Endotoxin Foetal Bovine Serum (FBS, Batch 91607)	9501	Harlan Sera Labs, Loughborough, UK
MEM non essential amino acids	11140-035	Invitrogen, Paisley, UK
NycoPrep 1.077 A	1113029	Axis-shield PoC AS, Oslo, Norway
Opti-MEM I Reduced Serum Medium	51985-042	GibcoBRL, Paisley, UK
Penicillin and Streptomycin		IAH Media Supplies, Compton, UK
Polymyxin B sulfate salt	P4932	Sigma Aldrich, Poole, UK
Red Blood Cell Lysis Buffer	R7757	Sigma Aldrich, Poole, UK
RPMI Medium (Phenol-Red Free)	1835-030	GibcoBRL, Paisley, UK
RPMI 1640 with Glutamax Medium	72400-021	GibcoBRL, Paisley, UK
Saline	F7124	Baxter Healthcare, Compton, UK
Trypan Blue	15250-061	GibcoBRL, Paisley, UK
Trypsin-EDTA	45300-019	GibcoBRL, Paisley, UK

2.1.5. Antibodies against human molecules

Antibody to	Clone	Isotype	Conjugated to	Working Dilution	Catalogue No.	Supplier
BDCA-1 (CD1c)	AD5-8E7	Mouse IgG2a	PE	1 in 100	130-090-508	Miltenyi Biotech, Auburn, USA
BDCA-2	AC144	Mouse IgG1	PE	1 in 50	130-090-511	Miltenyi Biotech, Auburn, USA
CCR5	CTC5	Mouse IgG1	APC	10 ul/2x10^5 cells	FAB1802A	R&D systems, Abingdon, UK
CCR7	150503	Mouse IgG2a	APC	10 ul/2x10^5 cells	FAB197A	R&D systems, Abingdon, UK
CD11c	3.9	Mouse IgG1	biotin	1 in 100	160-030	Ancell, Bayport, USA
CD11c	B-ly6	Mouse IgG1	PE	1 in 100	555392	BD biosciences PharMingen, Oxford, UK
CD123	9F5	Mouse IgG1	CyChrome	1 in100	551065	BD biosciences PharMingen, Oxford, UK
CD14	UCHM1	Mouse IgG2a	biotin	1 in 100	163-030	Ancell, Bayport, USA
CD16	3G8	Mouse IgG1	Purified		555404	BD biosciences PharMingen, Oxford, UK
CD161	DX12	Mouse IgG1	Purified		556079	BD biosciences PharMingen, Oxford, UK
CD19	HIB19	Mouse IgG1	APC	1 in 100	555415	BD biosciences PharMingen, Oxford, UK
CD27	M-T271	Mouse IgG1	PE	1 in 100	555441	BD biosciences PharMingen, Oxford, UK
CD28	CD28.2	Mouse IgG1	APC	1 in 100	559770	BD biosciences PharMingen, Oxford, UK
CD3	UCHT1	Mouse IgG1	PE	1 in 100	555333	BD biosciences PharMingen, Oxford, UK
CD3	UCHT1	Mouse IgG1	Purified	1 in 100	555333	BD biosciences PharMingen, Oxford, UK
CD32	AT10	Mouse IgG1	Purified		MCA1075XZ	Serotec Ltd, Oxford, UK
CD4	SK3	Mouse IgG1	PE-Cy5	1 in 100	555348	BD biosciences PharMingen, Oxford, UK
CD4	SK3	Mouse IgG1	PerCP	1 in 100	345770	BD biosciences PharMingen, Oxford, UK
CD4	SK3	Mouse IgG1	APC	1 in 100	345771	BD biosciences PharMingen, Oxford, UK
CD40	5C3	Mouse IgG1	Purified	1 in 100	555586	BD biosciences PharMingen, Oxford, UK
CD40	5C3	Mouse IgG1	FITC	1 in 100	555588	BD biosciences PharMingen, Oxford, UK
CD40	5C3	Mouse IgG1	PE	1 in 100	555589	BD biosciences PharMingen, Oxford, UK
CD45RA	HI100	Mouse IgG2b	CyChrome	1 in 50	555490	BD biosciences PharMingen, Oxford, UK
CD45RA	HI100	Mouse IgG2b	biotin	1 in 50	31262X	BD biosciences PharMingen, Oxford, UK
CD45RO	UCHL1	Mouse IgG2a	biotin	1 in 12.5	CBL 133B	Cymbus biotechnology, Hants, UK
CD45RO	UCHL1	Mouse IgG2a	CyChrome	1 in 25	555494	BD biosciences PharMingen, Oxford, UK
CD54	HA58	Mouse IgG1	PE	1 in 100	31625X	BD biosciences PharMingen, Oxford, UK
CD56	N901	Mouse IgG1	PC5	1 in 100	A07789 (PN IM2654)	Beckman Coulter, High Wycombe

Antibody to	Clone	Isotype	Conjugated to	Working Dilution	Catalogue No.	Supplier
CD45RO	UCHL1	Mouse IgG2a	CyChrome	1 in 25	555494	BD biosciences PharMingen, Oxford, UK
CD54	HA58	Mouse IgG1	PE	1 in 100	31625X	BD biosciences PharMingen, Oxford, UK
CD56	N901	Mouse IgG1	PC5	1 in 100	A07789 (PN IM2654)	Beckman Coulter, High Wycombe
CD69	FN50	Mouse IgG1	FITC	1 in 100	555530	BD biosciences PharMingen, Oxford, UK
CD8	SK1	Mouse IgG1	PerCP	1 in 100	345774	BD biosciences PharMingen, Oxford, UK
CD8	SK1	Mouse IgG1	APC	1 in 100	345775	BD biosciences PharMingen, Oxford, UK
CD8	HIT8a	Mouse IgG1	biotin	1 in 100	33292X	BD biosciences PharMingen, Oxford, UK
CD80 (BB1/B7-1)	BB1	Mouse IgM	biotin	1 in 100	33512X	BD biosciences PharMingen, Oxford, UK
CD80 (BB1/B7-1)	BB1	Mouse IgM	FITC	1 in 100	33514X	BD biosciences PharMingen, Oxford, UK
CD81	JS-81	Mouse IgG1	FITC	1 in 100	551108	BD biosciences PharMingen, Oxford, UK
CD81	1.3.3.22	Mouse IgG1	Purified	1 in 100	sc-7637	Santa Cruz Biotechnology, California, USA
CD81	1.3.3.22	Mouse IgG1	Purified	1 in 100	MS-502-PABX	Stratech Scientific, Soham, UK
CD81	JS-81	Mouse IgG1	Purified		555675	BD biosciences PharMingen, Oxford, UK
CD81-A		Mouse IgG1	Purified	1 in 100		Dr Arvind Patel, Glasgow, UK
CD81-B		Mouse IgG1	Purified	1in 100		Dr Arvind Patel, Glasgow, UK
CD83	HB15e	Mouse IgG1	PE	1 in 100	556855	BD biosciences PharMingen, Oxford, UK
CD83	HB15e	Mouse IgG1	FITC	1 in 100	556910	BD biosciences PharMingen, Oxford, UK
CD86	2331	Mouse IgG1	PE	1 in 100	555658	BD biosciences PharMingen, Oxford, UK
CD86	IT2.2	MousenIgG2b	biotin	1 in 100	33432X	BD biosciences PharMingen, Oxford, UK
CD95	DX2	Mouse IgG1	biotin	1 in 100	555672	BD biosciences PharMingen, Oxford, UK
CLA-1 (SR-BI)	25	Mouse IgG1	Purified	1 in 12.5	610883	BD biosciences PharMingen, Oxford, UK
$F(ab')_2$ fragment		Goat anti-mouse IgG	FITC	1 in 100	115-096-072	Jackson Immunoresearch, Baltimore, USA
$F(ab')_2$ fragment		Goat anti-rat IgG	FITC	1 in 100	112-096-072	Jackson Immunoresearch, Baltimore, USA
HLA-A, B, C	W6/32	Mouse IgG2a	Biotin	1 in 50	H200	Leinco Technologies, St. Louis, USA
HLA-A,B,C	G46-2.6	Mouse IgG1	PE	1 in 100	555553	BD biosciences PharMingen, Oxford, UK
HLA-DR	G46-6	Mouse IgG2a	PE	1 in 100	555812	BD biosciences PharMingen, Oxford, UK
IgG		Goat anti-Mouse IgG	Peroxidase	1 in 1000	115-035-164	Jackson Immuno research, West Grove, USA
IgG		Goat anti-Rat IgG	Peroxidase	1 in 1000	3010-05	Harlan, Oxon, UK
IgG1	MOPC-21	Mouse IgG1	FITC		555748	BD biosciences PharMingen, Oxford, UK

Antibody to	Clone	Isotype	Conjugated to	Working Dilution	Catalogue No.	Supplier
IgG1	MOPC-21	Mouse IgG1	PE		554680	BD biosciences PharMingen, Oxford, UK
IgG1	MOPC-21	Mouse IgG1	biotin		33812X	BD biosciences PharMingen, Oxford, UK
IgG1	MOPC-21	Mouse IgG1	Purified		555746	BD biosciences PharMingen, Oxford, UK
IgG1	MOPC-21	Mouse IgG1	APC		555751	BD biosciences PharMingen, Oxford, UK
IgG1/Fc			Purified		110-HG-100	R&D systems, Abingdon, UK
IgG2b	20116.11	Mouse IgG2b	Purified		MAB004	R&D systems, Abingdon, UK
IgG2b	dG9	Mouse IgG2b	FITC		556577	BD biosciences PharMingen, Oxford, UK
MHC class II	TDR31.1	Mouse IgG1	Biotin	1 in 50	131-030	Ancell, Bayport, USA
MICA/Fc chimera			Purified		1300-MA-050	R&D systems, Abingdon, UK
NKG2C	134522	Mouse IgG2b	Purified		MAB1381	R&D systems, Abingdon, UK
NKG2D	149810	Mouse IgG1	Purified		MAB139	R&D systems, Abingdon, UK
Penta-His		Mouse IgG1	Purified	2 ug/ml	34660	Qiagen, Crawley, UK
Penta-His		Mouse IgG1	Alexa Fluor 488	2 ug/ml	35310	Qiagen, Crawley, UK
Perforin	deltaG9	Mouse IgG2b	FITC	2.5 ul/million cells	556577	BD biosciences PharMingen, Oxford, UK
streptavidin			APC	1 in 600	554067	BD biosciences PharMingen, Oxford, UK
streptavidin			PerCP	1 in 600	551419	BD biosciences PharMingen, Oxford, UK
streptavidin			FITC	1 in 600	SA1001	Caltag, Burlingame, USA

2.1.6. Antibodies against mouse molecules

Antibody to	Clone	Isotype	Conjugated to	Working Dilution	Catalogue No.	Supplier
1-A/1-E	2G9	Rat IgG2a	biotin	1 in 100	553622	BD biosciences PharMingen, Oxford, UK
CD11c	HL3	Armenian Hamster IgG1	PE	1 in 200	553802	BD biosciences PharMingen, Oxford, UK
CD11c	HL3	Armenian Hamster IgG1	FITC	1 in 200	553801	BD biosciences PharMingen, Oxford, UK
CD11c	HL3	Armenian Hamster IgG1	APC	1 in 200	550261	BD biosciences PharMingen, Oxford, UK
CD4 (L3T4)	RM4-5	Rat IgG2a	FITC	1 in 100	553047	BD biosciences PharMingen, Oxford, UK
CD40	HM40-3	Armenian Hamster IgM	Purified		553721	BD biosciences PharMingen, Oxford, UK
CD40	3/23	Rat IgG2a	biotin	1 in 100	553789	BD biosciences PharMingen, Oxford, UK
CD45R/B220	RA3-6B2	Rat IgG2a	PE	1 in 100	553090	BD biosciences PharMingen, Oxford, UK
CD49b/Pan-NK	DX5	Rat IgM	FITC	1 in 100	553857	BD biosciences PharMingen, Oxford, UK
CD54 (ICAM-1)	3E2	Armenian Hamster IgG1	biotin	1 in 100	01542D	BD biosciences PharMingen, Oxford, UK
CD8 (Ly-2)	53-6.7	Rat IgG2a	FITC	1 in 100	553031	BD biosciences PharMingen, Oxford, UK
CD80 (B7-1)	16-10A1	Armenian Hamster IgG1	biotin	1 in 100	553767	BD biosciences PharMingen, Oxford, UK
CD81	Eat2	Armenian Hamster IgG1	PE	5 ul	559519	BD biosciences PharMingen, Oxford, UK
CD86 (B7-2)	GL1	Rat IgG2a	biotin	1 in 100	09272D	BD biosciences PharMingen, Oxford, UK
H-2Kb	AF6-88.5	Mouse IgG2a	biotin	1 in 100	553568	BD biosciences PharMingen, Oxford, UK
IgG		Anti-Armenian Hamster IgG	FITC		127-095-160	Jackson Immunoresearch, Baltimore, USA
IgG	A19-3	Armenian Hamster IgG	Purified		553972	BD biosciences PharMingen, Oxford, UK
IgG1	A19-3	Armenian Hamster IgG1	PE	5 ul	553972	BD biosciences PharMingen, Oxford, UK
IgG1	G235-2356	Armenian Hamster IgG1	APC	1 in 200	553956	BD biosciences PharMingen, Oxford, UK
IgM	G235-1	Armenian Hamster IgM	Purified		553957	BD biosciences PharMingen, Oxford, UK
IgG1		Mouse IgG1	APC	10 ul/million cells	MG105	Caltag, Burlingame, USA

2.1.7. Antibodies against the HCV E2 protein

Anti-E2 mAbs	Isotype	Provided by
H53	Mouse IgG1	Dr Jean Dubuisson (Institute de Biologie de Lille & Institut Pasteur de Lille, Lille Cedex, France)
H50	Mouse IgG1	Dr Jean Dubuisson (Institute de Biologie de Lille & Institut Pasteur de Lille, Lille Cedex, France)
H33	Mouse IgG1	Dr Jean Dubuisson (Institute de Biologie de Lille & Institut Pasteur de Lille, Lille Cedex, France)
H48	Mouse IgG1	Dr Jean Dubuisson (Institute de Biologie de Lille & Institut Pasteur de Lille, Lille Cedex, France)
1/39	Rat IgG1	Dr Jane McKeating (University of Reading, UK)
6/53	Rat IgG1	Dr Jane McKeating (University of Reading, UK)
3/11	Rat IgG1	Dr Jane McKeating (University of Reading, UK)
9/27	Rat IgG1	Dr Jane McKeating (University of Reading, UK)
9/86a	Rat IgG1	Dr Jane McKeating (University of Reading, UK)
AP33	Mouse IgG1	Dr Arvind Patel (MRC Virology Unit, Institute of Virology, Glasgow, UK)
ALP98	Mouse IgG1	Dr Arvind Patel (MRC Virology Unit, Institute of Virology, Glasgow, UK)
ALP266	Mouse IgG1	Dr Arvind Patel (MRC Virology Unit, Institute of Virology, Glasgow, UK)
AP33 Fab-FLUOS	Mouse IgG1	Dr Arvind Patel (MRC Virology Unit, Institute of Virology, Glasgow, UK)

2.1.8. Plasmids

Protein	Genotype	Plasmid	Vector	Selection marker	Provided by
$E2_{660}$ H77c	1a	pCDNA10H	pCDNA3.1/Zeo(+)	Ampicillin	Dr Arvind Patel (MRC Virology Unit, Institute of Virology, Glasgow, UK)
$E2_{660}$ Gla	1a	pCTF10	pCDNA3.1/Zeo(+)	Ampicillin	Dr Arvind Patel (MRC Virology Unit, Institute of Virology, Glasgow, UK)
$E2_{660}$ C3	1a	pCTFH3	pCDNA3.1/Zeo(+)	Ampicillin	Dr Arvind Patel (MRC Virology Unit, Institute of Virology, Glasgow, UK)
$E2_{661}$ BK	1b	BK	pVIjnsTPA	Kanamycin	Dr Alessandra Vitelli (IRBM, Pomezia, Italy)
$E2_{661}$ 1A14	1a	UKN14.42.5	pCR3.1	Ampicillin	Dr Alex Tarr (Division of Microbiology and infectious diseases, University of Nottingham, UK
$E2_{661}$ 1B12	1b	UKN1B12.6.18	pCR3.1	Ampicillin	Dr Alex Tarr (Division of Microbiology and infectious diseases, University of Nottingham, UK
GFP		pCDNA-GFP	pCDNA	Ampicillin	Dr Mike Flint (University of Reading, UK)

2.2. Cell preparation

2.2.1. Maintenance of cell lines

Hepatocyte cell lines (Hep16, Hep3B, Huh-7, and HepG2 cells) were cultured in Dulbecco's Modified Eagle Medium with Glutamax-1 (DMEM) supplemented with 7 % foetal calf serum (FCS) and 1 μg/ml penicillin and streptomycin (DMEM regular medium) and Molt-4 cells were cultured in RPMI supplemented with 10 % FCS and 1 μg/ml penicillin/streptomycin (RPMI regular medium). They were maintained at 37 °C in 5 % CO_2 and subcultured, when they were confluent (approximately twice a week). Adherent cells were detached using 0.05 % trypsin/0.02 % ethylene-diamino-tetra-acetate (EDTA) solution.

2.2.2. Isolation of peripheral blood mononuclear cells

Blood samples were obtained from blood bags purchased from the North London Blood Transfusion Service (Colindale, UK) or drawn with informed consent from healthy adult donors. Ethical approval for this study was obtained from the institutional review board (the Edward Jenner Institute for Vaccine Research, UK).

Human PBMCs were isolated from heparinised blood using Histopaque density gradient centrifugation. Blood was diluted 1:1 in saline. 25 ml diluted blood was layered onto 15 ml pre-warmed Histopaque-1077 and centrifuged at 1600 rpm at room temperature for 30 minutes in a Sorvall RT7 centrifuge (Sorvall, Stevenage, UK) with the brake off. PBMCs were harvested from the interface and washed three times in 50 ml saline. After the third wash, cells were resuspended in 50 ml RPMI regular medium. An aliquot of cells was diluted in trypan blue, and viable cells were counted using a haemacytometer.

Some PBMCs were resuspended in freezing solution [10 % (v/v) dimethyl sulphoxide (DMSO) in FCS] and stored in liquid nitrogen until use, but cells were typically used fresh.

2.2.3. Production of monocyte-derived dendritic cells

Monocyte-derived DCs were generated from $CD14^+$ cells that were isolated from PBMCs by positive selection. PBMCs were pelleted at 1300 rpm for 5 minutes in a Sorvall RT7 centrifuge, and were incubated with anti-CD14 MACS MicroBeads at a concentration of 5 µl beads per 10^7 PBMCs for 10 minutes on ice. Cells were washed twice with 50 ml of MACS buffer [RPMI, 2 % (v/v) FCS, 2 mM EDTA], then were resuspended in 6 ml MACS buffer and passed through a 40 µm cell strainer to remove clumps of cells. MACS LS columns were equilibrated with 3 ml of MACS buffer per column, then 3 ml of cell suspension was added per column. The columns were placed in a magnetic field. Magnetically labelled $CD14^+$ cells were retained in the columns, while unbound cells passed through them. The columns were washed three times with 3 ml MACS buffer to remove all unbound cells. The columns were then removed from the magnetic field, and $CD14^+$ cells were flushed out from each column in 3 ml MACS buffer using a plunger. The cells were then passed over the columns again to increase the purity. This time, after the third wash, $CD14^+$ cells were eluted from the columns in 3 ml of DC culture medium [RPMI supplemented with 10 % (v/v) low endotoxin foetal bovine serum (FBS), 1 ug/ml penicillin and streptomycin, and 100 U/ml polymyxin B] per column.

Eluted $CD14^+$ cells were counted. Routinely, ~10 % of the starting PBMCs were recovered as $CD14^+$ cells after purification. $CD14^+$ cells were cultured at 5 x 10^6 cells/well in 6-well plates with DC culture medium supplemented with 50 ng/ml IL-4

and 200 ng/ml human granulocyte-macrophage-colony stimulating factor (GM-CSF) at 37 °C in 5 % CO_2 for 6 to 7 days, to allow monocytes to differentiate into DCs. On the third day of culture, a quarter of the old medium was replaced with fresh DC culture medium supplemented with IL-4 and GM-CSF.

Many cells were lost during the culture and 30-70 % of starting $CD14^+$ cells were recovered on the day of harvest. After harvesting, monocyte-derived DCs were stained with a phycoerythrin (PE)-conjugated anti-CD11c mAb and analysed by flow cytometry as described below to check their purity. Routinely, more than 95 % of viable cells harvested expressed CD11c.

Monocyte-derived DCs were typically used fresh, but they were sometimes resuspended in freezing solution and kept in liquid nitrogen until use. Prior to use, frozen cells were washed once in 50 ml of the appropriate buffer or media.

2.2.4. Purification of different PBMC subsets

In some experiments, T cells, NK cells, B cells, monocytes, myeloid DCs or plasmacytoid DCs were purified from PBMCs using MACS MicroBeads cell isolation kits and LS MACS columns, following the manufacturer's instructions. Where cells were positively selected, the desired cells were retained in the column and eluted after removing the column from the magnetic field as described for $CD14^+$ cell isolation in section 2.2.3. Where cells were negatively selected, desired cells were unlabelled and ran through the column in the magnetic field. Thus the flow-through was collected as a negatively selected fraction which contained purified cells.

T cells were isolated either by a positive or negative selection method. In the positive selection method, PBMCs were incubated with anti-CD3 MACS MicroBeads. In the

negative selection method, PBMCs were incubated with a cocktail of hapten-conjugated anti-CD11b, CD16, CD19, CD36 and CD56 mAbs followed by an anti-hapten mAb conjugated to microbeads. After depletion of B cells, monocytes, NK cells, DCs, early erythroid cells, platelets and basophils, approximately 60 % of the starting PBMCs were recovered in a flow-through fraction containing T cells. The purity of T cells isolated in this way was analysed by staining cells with a PE-conjugated anti-CD3 mAb and carrying out flow cytometry. 81-89 % of the cells in the lymphocyte gate expressed CD3.

NK cells were purified by a negative selection method. PBMCs were incubated with a cocktail of hapten-conjugated anti-CD3, CD14, CD19, CD36 and IgE mAbs followed by an anti-hapten mAb conjugated to microbeads to deplete T cells, B cells and myeloid cells. Alternatively, PBMCs were incubated with a cocktail of biotin-conjugated monoclonal antibodies against CD3, CD4, CD14, CD15, CD19, CD36, CD123 and Glycophorin A followed by an anti-biotin mAb conjugated to microbeads to deplete T cells, B cells, DCs, monocytes, granulocytes and erythroid cells. Approximately 9 % of starting PBMCs were recovered in the final fraction containing NK cells. The purity of the NK cells was determined by staining cells with a PC5-conjugated anti-CD56 mAb and carrying flow cytometry. 58-74 % of the cells in the lymphocyte gate expressed CD56.

B cells were also isolated by a negative selection method. PBMCs were incubated with FcR blocking reagent and a cocktail of hapten-conjugated anti-CD2, CD4, CD11b, CD16, CD36 and IgE mAbs followed by an anti-hapten mAb conjugated to microbeads to deplete T cells, NK cells, myeloid cells, basophils, platelets and early erythroid cells.

Approximately 7 % of starting PBMCs were obtained in the final fraction containing B cells. The purity of the B cells was determined by staining cells with an allophycocyanin (APC)-conjugated anti-CD19 mAb and carrying out flow cytometry. 56 % of the cells in the lymphocyte gate expressed CD19.

Myeloid DCs were purified using two magnetic separation steps. In the first step, PBMCs were incubated with a FcR blocking reagent, anti-CD19 MACS MicroBeads, and a biotin conjugated anti-CD1c (BDCA-1) mAb. CD1c-expressing B cells were magnetically labelled with the anti-CD19 MACS MicroBeads; these were then depleted by using MACS columns. In the second step, the B cell-depleted flow-through fraction was incubated with anti-biotin MACS MicroBeads so that the remaining $CD1c^+$ cells were magnetically labelled. $CD1c^+$ cells were then positively selected by passage through another column. Approximately 1 % of starting PBMCs were recovered from the final fraction and the purity of myeloid DCs was determined by staining cells with a PE-conjugated anti-BDCA-1 mAb and carrying out flow cytometry. 42-82 % of the cells in the lymphocyte gate expressed BDCA-1^+.

Plasmacytoid DCs were purified by a positive selection method. PBMCs were incubated with a FcR blocking reagent and anti-BDCA-4 MACS MicroBeads. The positively-selected fraction was enriched for BDCA-4^+ plasmacytoid DCs. Approximately 0.2 % of starting PBMCs were recovered in the flow-through fraction containing plasmacytoid DCs. The purity of plasmacytoid DCs was determined by staining cells with a CyChrome-conjugated anti-CD123 Ab and a PE-conjugated BDCA-2 mAb. 54-96 % of the cells in the lymphocyte gate were plasmacytoid DCs.

2.3. Analysis of CD81 and SR-BI expression on cells

PBMCs (1 x 10^6/well), monocyte-derived DCs (5 x 10^5 - 1 x 10^6/well) and cell lines (2-5 x 10^5/well) were pelleted in a V-bottomed 96-well plates by spinning the plate at 1300 rpm for 5 minutes in a Sorvall RT7 centrifuge. Cells were stained with mAbs against cell surface markers, incubating the mAbs with the cells for 30 minutes - 1 hour at 4 °C. All mAbs were diluted in 50-100 µl fluorescence activated cell sorting (FACS) buffer [2 % (v/v) FCS, 0.2 % (w/v) sodium azide in phosphate buffered saline (PBS)] at the concentrations indicated in section 2.1.5.

Cells were firstly stained with a fluoresecin isothiocyanate (FITC)-conjugated anti-CD81 mAb (clone JS-81 or 2.3.3.22), anti-SR-BI mAbs (clones 25, 3D5 or 6B8) or an isotype-matched control mAb. Anti-SR-BI mAbs were detected using 50 µl FITC-conjugated anti-mouse IgG and IgM $F(ab')_2$ at a 1:100 dilution in FACS buffer added for 1 hour at 4 °C. PBMC subsets were identified by co-staining with the following mAbs: PE-conjugated anti-BDCA-1, PE-conjugated anti-BDCA-2, PE-conjugated anti-CD3, peridinin chlorophyll protein (PerCP)/APC-conjugated anti-CD4, PerCP-conjugated anti-CD8, biotin-conjugated anti-CD14, APC conjugated anti-CD19, PE-conjugated anti-CD27, 5PC-conjugated anti-CD56, biotin/CyChrome-conjugated anti-CD45RA, biotin/CyChrome-conjugated anti-CD45RO and CyChrome-conjugated anti-CD123 mAb. Biotin-conjugated mAbs were detected with 50 µl APC-conjugated streptavidin or PerCP-conjugated streptavidin which was diluted 1:600 in FACS buffer; they were incubated with the cells for 15 minutes at 4 °C. The combinations of mAbs used to identify each PBMC subset are described in Table 2.1. Cells were washed 3 times between all steps, each wash involving spinning the plates at 1300 rpm for 5 minutes in a Sorvall RT7 benchtop centrifuge, flicking out the supernatant and resuspending the cells in 200 µl FACS buffer. In some experiments, cells were fixed in

Table 2.1. The combinations of monoclonal antibodies used to identify PBMC subsets.

Subsets	mAb (FL2)[1]	mAb (FL3)[2]	mAb (FL4)[3]
$CD56^{dim}$ NK cells	CD3-PE	CD56-PC5	-
$CD56^{high}$ NK cells	CD3-PE	CD56-PC5	-
NT cells	CD3-PE	CD56-PC5	-
Myeloid DCs	BDCA-1-PE	-	CD19-APC
Plasmacytoid DCs	BDCA-2-PE	CD123-CyChrome	-
Granulocytes	BDCA-2-PE	CD123-CyChrome	-
Monocytes	-	-	CD14-biotin[4]
Naïve B cells	CD27-PE	-	CD19-APC
Memory B cells	CD27-PE	-	CD19-APC
$CD4^+$ $CD45RA^+$ T cells	CD3-PE	CD45RA-biotin[5]/CyChrome	CD4-APC
$CD4^+$ $CD45RA^+$ T cells	CD3-PE	CD4-PerCP	CD45RA-biotin[4]
$CD4^+$ $CD45RO^+$ T cells	CD3-PE	CD45RO-biotin[5]/CyChrome	CD4-APC
$CD4^+$ $CD45RO^+$ T cells	CD3-PE	CD4-PerCP	CD45RO-biotin[4]
$CD8^+$ $CD45RA^+$ T cells	CD3-PE	CD45RA-biotin[5]/CyChrome	CD8-APC
$CD8^+$ $CD45RA^+$ T cells	CD3-PE	CD8-PerCP	CD45RA-biotin[4]
$CD8^+$ $CD45RO^+$ T cells	CD3-PE	CD45RO-biotin[5]/Cychome	CD8-APC
$CD8^+$ $CD45RO^+$ T cells	CD3-PE	CD8-PerCP	CD45RO-biotin[4]

[1]Fluorochrome-conjugated monoclonal antibody (mAb) which can be detected in the FL2 channel on a FACSCalibur™ flow cytometer.

[2]Fluorochrome-conjugated mAb which can be detected in the FL3 channel on a FACSCalibur™ flow cytometer.

[3]Fluorochrome-conjugated mAb which can be detected in the FL4 channel on a FACSCalibur™ flow cytometer.

[4]Biotin-conjugated mAbs that were detected with APC-streptavidin.

[5]Biotin-conjugated mAbs that were detected with PerCP-streptavidin.

fixing buffer [2 % (v/v) formaldehyde in PBS] prior to analysis. Data was acquired using a FACSCalibur™ flow cytometer (Becton Dickinson) and analysed using CellQuest™ software (Becton Dickinson).

2.4. Production and purification of soluble HCV E2 proteins

2.4.1. Plasmid preparation

Plasmids encoding truncated HCV E2 proteins, encompassing aa 364 - aa 660 ($E2_{660}$) or aa 364 - aa 661 ($E2_{661}$), were used to produce soluble E2 proteins from genotype 1a or 1b viruses (section 2.1.8/Fig. 2.1). The E2 sequences expressed in all constructs encode the E2 signal sequence (aa 364-383), which is cleaved during polyprotein translation, leaving a mature protein comprising aa 384-660/661 of E2. All truncated E2 proteins carried six histidine residues at the C-terminus.

Plasmid pCDNA-GFP (Fig. 2.1) was used to allow determination of transfection efficiencies.

Competent *Escherichia coli* DH5α™ were transformed with these plasmids by a standard heat-shock method. Briefly, 1-10 ng of plasmid DNA in 1-5 μl volume was added to 50 μl of competent cells in Eppendorf tubes and incubated on ice for 30 minutes. Cells were given a heat-shock for 20 seconds at 37 °C before they were further incubated on ice for 2 minutes. 0.95 ml Luria-Bertani (LB) medium was then added, and the cells were incubated for one hour at 37 °C in an InnOva 4230 shaking incubator (New Brunswick Scientific, Edison, MJ, USA) at 225 rpm to allow the expression of the antibiotic resistance genes.

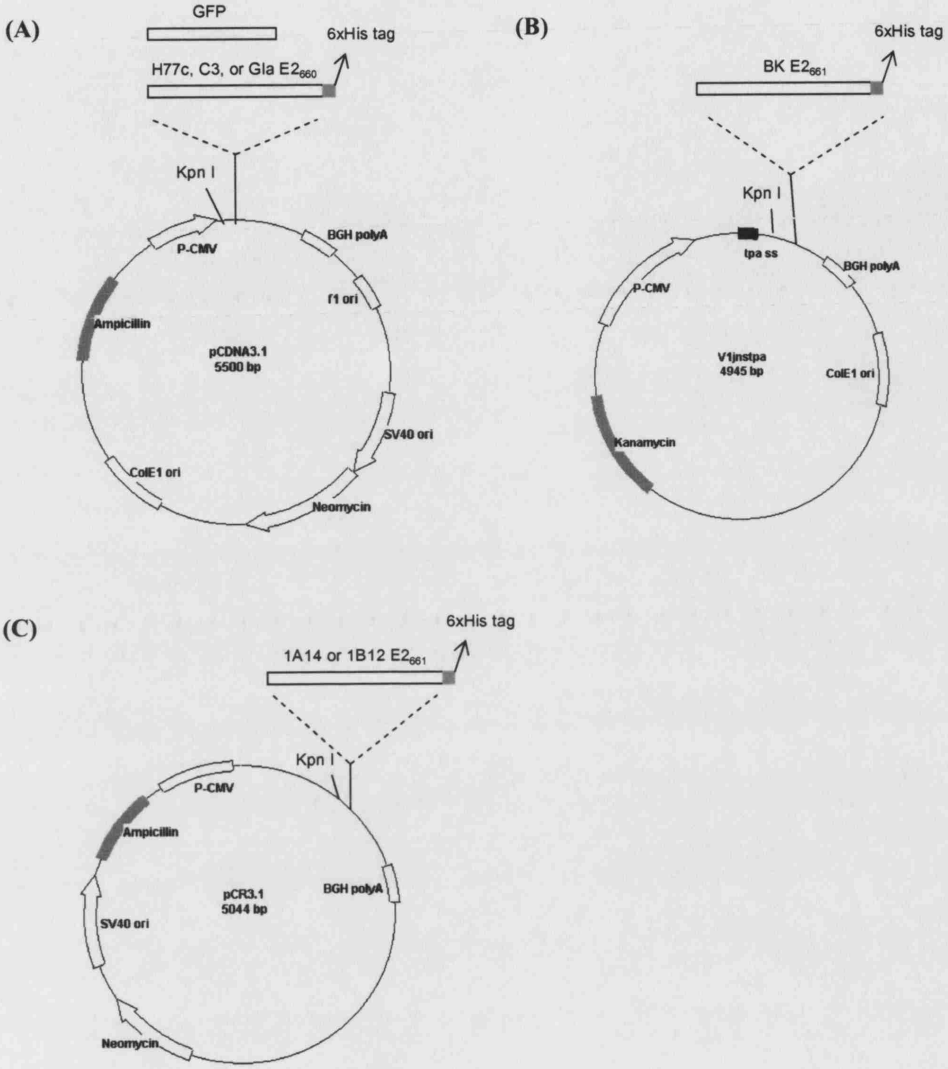

(A)
GFP
6xHis tag
H77c, C3, or Gla E2660
Kpn I
P-CMV
BGH polyA
f1 ori
Ampicillin
pCDNA3.1
5500 bp
SV40 ori
ColE1 ori
Neomycin
(B)
6xHis tag
BK E2661
Kpn I
tpa ss
BGH polyA
P-CMV
V1jnstpa
4945 bp
ColE1 ori
Kanamycin
(C)
6xHis tag
1A14 or 1B12 E2661
Kpn I
P-CMV
Ampicillin
pCR3.1
5044 bp
BGH polyA
SV40 ori
Neomycin

Figure 2.1. Expression plasmids used in this project.

The H77c, chimera 3 (C3) and Glasgow (Gla) $E2_{660}$ genes and the green fluorescent protein (GFP) gene were expressed from the pCDNA3.1 vector (A). The BK $E2_{661}$ gene was expressed from the V1jnstpa vector (B). The 1A14 or 1B12 $E2_{661}$ genes were expressed from the pCR3.1 vector (C). The sequence encoding a 6 x histidine tag (6xHis tag) at the end of each E2 gene is marked with a green box. The position of the Kpn I restriction enzyme site in each plasmid is indicated. The following are also indicated in the diagrams of the vectors: P-CMV, cytomegalovirus promoter; BGH polyA, bovine growth hormone polyadenylation site; tpa ss, TPA signal sequence; f1 ori, f1 origin of replication; SV40 ori, SV40 origin of replication; ColE1 ori, ColE1 origin of replication; Neomycin, neomycin resistance gene; Kanamycin, kanamycin resistance gene; Ampicillin, ampicillin resistance gene.

Transformed bacteria were selected by growth at 37 °C overnight on LB agar plates that contained appropriate antibiotics (100 μg/ml ampicillin or 50 μg/ml kanamycin). Single colonies were picked and expanded by growth in 10 ml LB containing appropriate antibiotics at 37 °C for 4-6 hours. Expanded bacteria were diluted 1:100 into 100 ml LB media containing appropriate antibiotics and incubated at 37 °C overnight in an InnOva 4230 shaking incubator at 225 rpm. 500 μl of transformed bacteria was mixed with 500 μl glycerol solution [1:1 (v:v) mixture of glycerol and H_2O] to make glycerol stocks that were stored at -20 °C.

Plasmids were purified using either a plasmid maxi kit or a GenElute high performance plasmid maxiprep kit following the manufacturer's instructions. Pelleted DNA was resuspended in 1 x TE buffer [0.5M EDTA, 1M Tris(hydroxymethyl) aminomethane (Tris) in H_2O at pH7.4] and stored in aliquots at -20 °C until use. Plasmid DNA was quantified using a GeneQuant Pro UV spectrophotometer (Amersham Pharmacia Biotech, Cambridge, UK). The identity of each plasmid was confirmed by restriction enzyme digestion. 1 μg of plasmid DNA was digested with Kpn I for 1 hour at 37 °C, then the products were run on 1 % agarose gels together with a 1 kb DNA ladder, and visualised under UV light (Jencons, Forest Row, UK).

2.4.2. Transfection of 293T/293FT cells and extraction of E2 proteins

293T cells were grown in DMEM regular medium. They were maintained at 37 °C in 5 % CO_2 and subcultured using 0.05 % trypsin/0.02 % EDTA solution to detach the cells when they were confluent (approximately twice a week). 293FT cells were grown in DMEM supplemented with 10 % FCS, penicillin and streptomycin, 0.1 mM non-

essential amino acids, and 0.5 mg/ml geneticin (293FT medium). Cells were maintained and subcultured in the same way as 293T cells.

Soluble truncated E2 proteins were produced by transfecting either 293T cells or 293FT cells with E2-encoding plasmids.

Plasmids were introduced into 293T cells using the GeneJuice transfection reagent. Cells were plated into 140 mm Nunclon™ dishes and maintained in 293T medium until they were 50-80 % confluent. Routinely, 10 dishes were prepared to transfect 293T cells with one plasmid. On the day of transfection, the cells were washed in saline, then 12 ml Opti-MEM supplemented with 2 % FCS and penicillin/streptomycin was added per plate. A master-mix of transfection reagents was prepared for each plasmid transfection. 400 µl of Opti-MEM supplemented with 2 % FCS and penicillin/streptomycin and 12 µl of the GeneJuice™ transfection reagent per plate were mixed by vortexing and incubated at room temperature for 5 minutes. 4 µg per plate of the plasmids encoding $E2_{660/661}$ or green fluorescent protein (GFP) were added to the transfection reagents. The transfection reagents were mixed gently by inverting the tubes several times, then incubated at room temperature for 5-15 minutes. 400 µl of master-mix was then added into cells in 12 ml of Opti-MEM, and the plates were gently agitated several times to distribute the plasmids evenly across the surface of the cells.

293FT cells were transfected using calcium phosphate. Cells were plated into 6-well cell culture plates and maintained in 293FT medium (3 ml/well) until they were 40 % confluent. 3 plates were prepared to transfect 293FT cells with one plasmid. For each plate to be transfected, 3 µg of the plasmids encoding $E2_{660/661}$ or GFP were mixed with 120 µl water, then 12 µl of 2.5 M $CaCl_2$ was added, and they were mixed gently by pipetting up and down. The DNA mix was added dropwise to 120 µl 2 x hepes-buffered

saline (heBS) while bubbling the heBS. The mix was votexed for a few seconds, and incubated for 20 minutes at room temperature. The precipitate was distributed dropwise over the cells, and the plate was agitated gently.

After a 72 hour incubation at 37 °C in 5 % CO_2, culture supernatants were pooled and supplemented with protease inhibitor cocktail tablets at 1 tablet/50 ml. The supernatants were concentrated approximately 40-fold using Centricon or Centriprep filter devices, following the manufacturers' instructions. Briefly, up to 80 ml of supernatant was added to a Centricon and centrifuged at 3000 rpm for 20-40 minutes at 4 °C in a J6-MC centrifuge (Beckman, High Wycombe, UK). The filter unit that retains proteins was inverted and centrifuged at 2000 rpm for 2-5 minutes at 4 °C in a J6-MC centrifuge. Alternatively, up to 17 ml of supernatant was added to a Centriprep and centrifuged at 3000 g in a J6-MC centrifuge for 2 hours at 4 °C. Concentrated supernatants were collected and stored at -20 °C until use.

To extract intracellular E2 proteins, 293T cells were harvested from 10 dishes by vigorous pipetting in saline and pelleted by centrifugation in a Sorvall RT7 centrifuge at 1400 rpm for 5 minutes. The pelleted cells were lysed by freezing and thawing three times. 1 ml protein extraction buffer (0.5 M Tris/HCl, 0.15 M NaCl, plus 2 protease inhibitor tablets per 100 ml at pH8.8) was added and the cells were sonicated in an ice-cold water bath for 3 minutes. Cell debris was removed by centrifugation at 1400 rpm in a Centrifuge 5415C (Eppendorf, Hamburg, Germany) for 2 minutes and the supernatants, which contained cell-associated E2 proteins, were aliquoted and stored at -20 °C until use.

2.4.3. Purification of soluble E2 proteins by Ni^{++} column chromatography

E2 proteins were purified from concentrated supernatants using Ni^{++} columns, following the manufacturer's instructions. The columns were equilibrated with 10 ml of binding buffer (10 mM imidazole in PBS at pH7.4), then concentrated supernatants (approximately 1-5 ml) were applied onto them. After washing with 10 ml of binding buffer, bound proteins were eluted from the columns using 5 ml of elution buffer (1 M imidazole in PBS at pH7.4) and collected in 1 ml fractions. All fractions were collected. These were tested for the presence of E2 proteins by Western blotting, as described below. Positive fractions were dialysed against PBS overnight, using slide-A-Lyzer dialysis cassettes, changing the PBS at least twice. The total protein concentration was determined using a micro protein determination kit, following the manufacturer's instructions. Briefly, 50 µl of protein sample was mixed with 2.5 ml of protein dye solution that contains brilliant blue G (Coomassie blue). A standard curve was made using serially diluted human albumin (original concentration was 30 mg/dl) and PBS as a blank. The optical density of the blue-coloured protein dye complex was measured at 595 nm using a GeneQuant Pro spectrophotometer. E2 proteins were stored in aliquots at -20 °C until use.

2.4.4. HPLC separation of soluble E2 proteins

Gel filtration chromatography was performed on a high pressure liquid chromatography (HPLC) Waters Separation module 2695 (Waters, Elstree, UK) using a TSK G4000PWXL (7.8 by 300 mm) column as described by Flint *et al.* (Flint *et al.*, 2000). 20-50 µg purified E2 protein was applied to the column in a volume of 50 µl. PBS was used as a solvent at a flow rate of 300 µl/min. 600 µl-fractions were collected at 2

minute intervals after 10 minutes. The absorbance was monitored at 214 and 280 nm using a Waters 2487 Dual Lambda Absorbance Detector (Waters).

2.5. Analysis of soluble E2 proteins by Western blotting and EIA

2.5.1. Detection of soluble E2 proteins by SDS-PAGE and Western blotting

E2 proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 10 % gels under non-reducing conditions together with molecular weight (MW) markers. The 10 % resolving gel [5 ml of 30 % acrylamide/0.8 % bisacrylamide, 3.75 ml of 4 x TrisCl/SDS (1.5 M TrisCl, 0.4 % SDS (w/v) at pH8.8), 6.25 ml of distilled H_2O, 50 µl of 10 % ammonium persulphate (APS), 10 µl of N, N, N', N'-Tetramethylethylenediamine (TEMED)] was poured into a sandwich of two glass plates, overlaid with isopropanol and allowed to polymerise at room temperature. The isopropanol was removed and the 4 % stacking gel [0.665 ml of 30 % acrylamide/0.8 % bisacrylamide, 1.25 ml of 4 x TrisCl/SDS [0.5 M TrisCl, 0.4 % SDS (w/v) at pH6.8], 3.05 ml distilled H_2O, 25 µl of 10 % APS, 5 µl TEMED] was added and the combs inserted. Once the gels were polymerised, the combs were removed and the gels were transferred to an electrophoresis tank filled with 1 x Tris/glycine SDS running buffer.

Protein samples were diluted 1:3 in Laemmli sample buffer and denatured at 95 °C for 4 minutes. The samples and markers were then loaded and electrophoresis was performed using a Power Pac 300 (BioRad, Hemel Hempstead, UK) run at 90 V until the samples entered the resolving gel and then at 100 V until the samples reached the bottom of the gel.

To allow the total protein content of each lane to be visualised, some gels were stained with Coomassie brilliant blue stain solution for one hour at room temperature or overnight at 4 °C and destained in Coomassie brilliant destain solution.

The proteins in other gels were transferred to Hybond ECL nitrocellulose membrane using a semi-dry transfer method. Gels, thick filter papers and nitrocellulose membranes were equilibrated in transfer buffer [48 mM Tris, 39 mM glycine, 0.03 % SDS (w/v), 20 % methanol (v/v) in H_2O at pH9.2] for 10 minutes on a mini orbital SO5 shaker (Stuart scientific, Redhill, UK). Gels were then placed onto the membrane and sandwiched with thick filter paper. A current was passed through them at a constant 10 V for 30 minutes using a Trans-blot semi-dry (BioRad).

Prior to Western blotting, membranes were blocked in 5 % blocking reagent [5 % Marvel non-fat milk (w/v), 0.1 % Tween-20 (v/v) in PBS] for 1 hour at room temperature or overnight at 4 °C on a mini orbital SO5 shaker. They were then washed twice with washing buffer [0.1 % (v/v) Tween-20 in PBS], which was also used for washes between all the subsequent steps.

Proteins were detected using mAbs specific for E2 proteins (section 2.1.7) or an anti-Penta-His mAb. Anti-E2 mAbs were produced from hybridomas. Hybridoma supernatants were used neat and the anti-Penta-His mAb was used at 2 μg/ml, diluted in PBS with 5 % Marvel milk. They were incubated with the membrane for 1-1.5 hours at room temperature on a mini orbital SO5 shaker. Bound antibodies were detected using a horseradish peroxidase (HRP) conjugated secondary anti-mouse or anti-rat IgG Ab at a 1:1000 dilution in PBS with 5 % Marvel milk. They were incubated with the membranes for 45 minutes-1 hour at room temperature on a shaker. The membrane was

then treated with chemiluminescence ECL Western blotting detection reagents (solution 1 and 2 mixed at 1:1). Blots were exposed to autoradiography film for times ranging from 5 seconds to one minute, and the films were then developed using an X-ray film processor X-ograph Compact X4 (X-ograph imaging systems, Malmesbury, UK). Protein bands were quantified by acquiring the images of exposed film using a scanner (Epson perfection 3200 Photo) or BioRad Fluor-S MultiImager (BioRad) and the relative quantities of monomeric E2 in different preparations was determined using Quantity One software (Version 4.1.1, BioRad) as described by Roccasecca *et al.* (Roccasecca *et al.*, 2003).

2.5.2. Analysis of soluble $E2_{660}$ binding to GNA lectin and CD81 by EIA

Enzyme immunosorbent assays (EIA) were carried out as described by others (Flint *et al.*, 1999b; Lucas *et al.*, 2003). Flat-bottomed 96-well EIA plates (Immulon-II ELISA plates) were coated overnight at 4 °C with 50 µl GNA lectin (*Galanthus nivalis*) at 2 µg/ml in Tris buffered saline (TBS, 144mM NaCl, 25mM Tris base at pH7.5), or with recombinant glutathione S-transferase (GST)-CD81 fusion proteins that contained the LEL from human CD81 (hCD81; aa 116-202) or mouse CD81 (mCD81; aa 109-201) (Flint *et al.*, 1999b; Higginbottom *et al.*, 2000) (the plasmids encoding the GST-CD81 fusion proteins were obtained from Dr. Shoshana Levy, Stanford University, Stanford, USA, and proteins were expressed by Dr. Maria Montoya, The Edward Jenner Institute for Vaccine Research, UK) at 5-10 µg/ml in TBS. Plates were washed three times between all steps with 300 µl of TBS. Plates were blocked with 200 µl of 4 % Marvel in TBS for 30 minutes at room temperature. Two-fold dilutions of E2 were prepared in TBS (the highest concentration used was 2 µg/well of E2) and 50 µl of each dilution was added to triplicate wells. Plates were incubated for 2 hours at room temperature. As

a positive control, an anti-human CD81 mAb (clone 1.3.3.22) at 2 μg/ml in TBS was added to the hCD81-coated plates. Bound E2 was detected using anti-E2 mAb H53 or a Penta-His mAb: plates were incubated with 50 μl per well of neat hybridoma supernatant of mAb H53 or the anti-Penta-His mAb at 4 μg/ml in TBS for 1 hour at room temperature. H53, anti-Penta-His, and anti-CD81 mAbs were detected using 50 μl per well of a HRP-conjugated secondary anti-mouse IgG Ab diluted 1:1000 in TMTSS [0.5% (v/v) Tween 20, 4% (w/v) milk, 20% (v/v) FCS in TBS]. The secondary mAb was incubated for 1 hour at room temperature. HRP was detected using 50 μl per well of TMB substrate solution (1:1 mixture of tetramethylbenzidine (TMB) and H_2O_2) and colour development was allowed in darkness. The reaction was stopped by addition of 50 μl per well of 10 % sulphuric acid. The coloured product was detected by measuring absorbance values at 450 nm, using a SpectraMax 340 reader (Molecular Devices, Winnersh, UK).

2.5.3. Analysis of recognition of soluble E2 by different anti-E2 mAbs using an EIA

Flat-bottomed 96-well EIA plates were coated with GNA lectin and blocked as described in section 2.5.2. E2 was added to multiple wells at 5 μg per well in 50 μl TBS, and plates were incubated for two hours at room temperature. Bound E2 was then detected using a panel of anti-E2 mAbs (section 2.1.7): duplicate wells were incubated with neat hybridoma supernatants for one hour at room temperature. Anti-E2 mAbs were detected and analysed as described in section 2.5.2.

2.6. Analysis of HCV E2 protein binding to hepatocyte cell lines and PBMC subsets

2.6.1. Binding of soluble $E2_{660/661}$ to cells

In some experiments, PBMCs were incubated in 1 ml PBS supplemented with 5 % human serum for 10 minutes on ice to block Fc receptors prior to analysis of E2 binding. Cells were pelleted in V-bottomed 96-well plates by spinning the plate at 1300 rpm for 5 minutes in a Sorval RT7 centrifuge. The supernatant was discarded, and the pelleted cells in residual buffer were incubated with purified E2 proteins (70 μg or other amounts as indicated) for two hours at room temperature. Cells were then washed with 200 μl FACS buffer. Washing was repeated 3 times between all steps. Binding of E2 proteins was detected using mAb H53 (100 μl neat supernatant per well) followed by a FITC-conjugated anti-mouse IgG and IgM $F(ab')_2$ (50 μl of a 1:100 dilution in FACS buffer), each incubated with the cells for 1 hour at 4 °C. Alternatively, E2 proteins were detected using an Alexa fluor 488-conjugated anti-Penta-His mAb, adding 50 μl of antibody at a concentration of 2 μg/ml in FACS buffer per well. In order to determine the background staining level, some cells were incubated with detection mAbs only in the absence of E2. Different PBMC subsets were identified by co-staining with combinations of mAbs as described in section 2.3. E2 binding was detected using a FACSCalibur™ flow cytometer and analysed using CellQuest™ software.

2.6.2. Blocking of soluble $E2_{660/661}$ binding to cells

In experiments addressing the ability of anti-E2 mAb H33 (Deleersnyder *et al.*, 1997; Patel *et al.*, 2000) to block E2 binding to cells, 70 μg of soluble E2 protein was incubated with 34 μg of mAb H33 for 1 hour at room temperature prior to addition to

cells. $E2_{660}$ with/without mAb H33 was then added to 1 x 10^6 PBMCs or monocyte-derived DCs resuspended in 50 µl FACS buffer, and incubated for 2 hours at room temperature. E2 binding was detected using anti-E2 mAb H53 followed by a FITC-conjugated secondary mAb as described in section 2.6.1.

In experiments addressing the ability of anti-CD81 or anti-SR-BI mAbs to block E2 binding to cells, 1 x 10^6 PBMCs or monocyte-derived DCs resuspended in 50 µl FACS buffer were pre-incubated with 0.5 µg of anti-CD81 mAb (clone JS-81 or 1.3.3.22), 10 µg of anti-SR-BI mAb (clone 3D5), an isotype-matched control mAb or a combination of anti-CD81 and anti-SR-BI mAbs for one hour at 4 °C prior to the addition of E2 proteins. After washing the cells once with FACS buffer, 70 µg $E2_{660}$ was added, and this was incubated with cells in residual buffer for 2 hours at room temperature. E2 binding was then detected using purified mAb H53 directly labelled with Alexa Fluor 488, prepared as described below. 15 µl mAb mixture was incubated with E2 bound-cells in residual buffer for 1 hour at 4 °C.

2.6.3. Purification and labelling of anti-E2 mAb H53

Anti-E2 mAb H53 was purified from hybridoma supernatant on a HPLC Waters Separation module 2695 apparatus, using a HiTrap Protein G affinity column. The IgG protein content of the elute was monitored at 214 nm and 280 nm using a Waters 2487 Dual lambda Absorbance detector. The fractions which showed the highest protein content were pooled and the antibody concentration was quantified by measuring the optical density at 280nm using a GeneQuant Pro spectrophotometer. IgG at a concentration of 1mg/ml gives an A_{280} value of 1.35. Using this information, the concentration of purified H53 was calculated from the A_{280} value.

Purified mAb H53 was directly labelled with the fluorescent molecule Alexa fluor 488 using a Zenon Alexa fluor 488 mouse IgG1 labelling kit, following the manufacturer's direction. 0.8 μg of purified H53 was incubated with 5.36 μl of the Zenon mouse IgG labelling reagent (component A), which contained labelled goat Fab fragments specific for the Fc portion of mouse IgG antibody, for 5 minutes at room temperature. Then the labelling complex was incubated with 5.36 μl of the Zenon blocking reagent (component B), which contained non-specific mouse IgG, for another 5 minutes at room temperature. During the second incubation, the non-specific mouse IgG bound to excess Fab fragments (component A). Labelled H53 was prepared freshly for each experiment just before adding to the cells.

2.7. Sequence alignment

The protein sequences of the H77c and BK E2 proteins were obtained from the GenBank database. The GenBank accession no. for each E2 protein sequence is as follows: H77c strain-AF011753 and BK strain-M58335. The sequences of 1A14 and 1B12 $E2_{661}$ were obtained from Dr Alex Tarr (Division of Microbiology and Infectious Diseases, Nottingham University, UK). Multiple sequence alignment was carried out using a CLUSTAL W program (EMBL-European Bioinformatics Institute, http://www.ebi.ac.uk/clustalw/).

2.8. Analysis of the binding of other forms of E2 to cells

2.8.1. Binding of VLPs

VLPs produced in a baculovirus expression system (Clayton *et al.*, 2002; Owsianka *et al.*, 2001) were kindly provided by Dr Arvind Patel (MRC Virology Unit, Institute of

Virology, Glasgow, UK). Two types of VLP were used in this project: B45 VLPs expressing core proteins and E1E2 heterodimers from the infectious clone H77c and BCS6G VLPs expressing core and E1 from H77c and E2 from chimera 3 (C3) (Patel *et al.*, 2000). VLPs were kept at 4 °C and used within 1 week of production.

VLPs were sonicated for few seconds and mixed by pipetting up and down. Different volumes of H77c and C3 VLPs (5, 2, 0.2, and 0.02 µl from stock batches containing 10^{11} VLPs/ml) were incubated in V-bottomed 96-well plates with 1 x 10^6 cells in 50 µl FACS buffer per well for 2 hours on ice. VLP binding was then detected using 100 µl of AP33 Fab-FLUOS (a fluorescent-conjugated Fab fragment of anti-E2 mAb AP33) at a 1:200 dilution in FACS buffer or 100 µl of anti-E2 mAb H53 (neat hybridoma supernatant) followed by a FITC-conjugated anti-mouse IgG and IgM $F(ab')_2$ (50 µl of a 1:100 dilution in FACS buffer). Cells were washed 3 times between all steps as described previously. Different PBMC subsets were identified by co-staining with combinations of mAbs as described in section 2.3. VLP binding was detected using a FACSCalibur™ flow cytometer and analysed using CellQuest™ software.
In experiments addressing the ability of anti-E2 mAb H33 to block the binding of VLPs to MDDCs, 4 µl of VLPs were incubated with 35 µg of purified mAb H33 for 1 hour at room temperature prior to addition to the cells.

2.8.2. Binding of HCV pseudotyped particles

HCV pseudotyped particles (HCVpp) produced using a murine leukaemia virus (MLV) packaging system were kindly provided by Dr Anne Goffard, Dr Cécile Voisset and Dr Jean Dubuisson (Institute de Biologie de Lille & Institut Pasteur de Lille, Lille, France). Two types of pseudoparticles were used in this study: HCVpp expressing E1E2

heterodimers from the infectious HCV clone H77c and control particles without HCV glycoproteins (Contp) (Bartosch *et al.*, 2003a). HCVpp and Contp preparations were normalised on the basis of expression of MLV core proteins, which were detected by Western blotting using MLV anti-capsid antiserum (Bartosch *et al.*, 2003a).

1 x 10^6 cells (PBMCs, hepatocytes cell lines or Molt-4 cells) were incubated with 500 µl or 1 ml of HCVpp or Contp in 48-well plates for 2 hours on ice or at room temperature. Particle binding was detected using anti-E2 mAbs H48, H33 or H53 (100 µl neat hybridoma supernatant per well) followed by a FITC-conjugated anti-mouse IgG and IgM $F(ab')_2$ (50 µl of a 1:100 dilution in FACS buffer per well), each incubated for 1 hour on ice. Different PBMC subsets were identified by co-staining with combinations of mAbs as described in section 2.3. Cells were washed with FACS buffer 3 times between all steps. Particle binding was detected using a FACSCalibur™ flow cytometer and analysed using CellQuest™ software.

2.8.3. Binding of HCV virions

HCV virions were kindly supplied by Dr Christian Schuttler (Justus-Liebig-University, Giessen, Germany). Serum containing HCV virions was obtained with informed consent from an immunosuppressed patient infected with a genotype 1a virus. The patient's serum HCV titre was 2 x 10^7 IU/ml, as determined by real-time RT-PCR (Schuttler *et al.*, 2004). HCV virions that were free from lipoproteins and immunoglubulins were purified by gel exclusion chromatography.

PBMCs were incubated in a 96-well plate at 1 x 10^6 cells/well with 100 µl of virions per well for 2 hours at 4 °C. Virion binding was detected using anti-E2 mAb H53 (100 ul of

neat hybridoma supernatant per well) followed by a FITC-conjugated anti-mouse IgG and IgM $F(ab')_2$ (50 µl per well of a 1:100 dilution in FACS buffer), each incubated with the cells for 1 hour at 4 °C. In order to investigate virion binding to different PBMC subsets, cells were co-stained with mAbs against distinguishing cell surface markers as described in section 2.3. Cells were washed 3 times between all steps as described previously. Particle binding was detected using a FACSCalibur™ flow cytometer and analysed using CellQuest™ software.

2.9. Analysis of infection of cells by HCV pseudotyped particles using a luciferase assay

Both HCVpp and Contp (described in section 2.8.2) carried luciferase genes. Infection of cells by HCVpp and control particles was determined using a luciferase assay as described by Op De Beeck *et al.* (Op De Beeck *et al.*, 2004).

Briefly, Hep3B cells were plated in 12-well plates at a density of 8×10^4 cells per well. After overnight incubation at 37 °C in 5 % CO_2, the medium was removed from the cells. PBMC subsets were prepared on the day of infection and added to 12-well plates at the following cell numbers/well in a small volume of RPMI regular medium: (1) B cells at 5.5-10 x 10^5 cells/well, (2) T cells at 1-2 x 10^6 cells/well, (3) monocytes at 7-10 x 10^5 cells/well, and (4) monocyte-derived DCs at 5-50 x 10^4 cells/well.

500 µl - 1 ml of HCVpp or Contp were incubated with the cells for 3 hours at 37 °C in 5 % CO_2. The cells were then centrifuged at 1300 rpm for 5 minutes in a Sorvall RT7 benchtop centrifuge and the supernatant was removed. 2 ml DMEM or RPMI regular medium was added per well to Hep3B cells or PBMCs respectively. In order to activate PBMC subsets, the following stimuli were added: (1) Ig beads at 3.3 ul/well and IL-4 at

12.5 ng/ml for B cells, (2) 10 μg/ml phytohemagglutinin (PHA) for T cells, (3) 10 ng/ml lipopolysaccharide (LPS) for monocytes and (4) 10 ng/ml LPS and 100 ng/ml of IL-4 for monocyte-derived DCs. The cells were then incubated for 72 hours at 37 °C in 5 % CO_2. On day 4, the cells were centrifuged at 1300 rpm for 5 minutes in a Sorvall RT7 benchtop centrifuge and the supernatant was discarded.

Luciferase expression within the cells was then determined by using a Luciferase Assay System. Cells were lysed with 120 μl 1 x lysis buffer, then 4 μl of each lysate was mixed with 20 μl luciferase substrate in 96-well microtitre plates (Corning). Luminescence was quantified on a Wallac Victor^2 1420 multilabel counter (PerkinElmer, Beaconsfield, UK). Results were expressed as counts per second (cps).

2.10. Analysis of the effects of CD81 cross-linking on the response of cells to different stimuli

2.10.1. Stimulation of cells by plate-bound antibodies

2.10.1.1. Preparation of antibody-coated plates

To immobilize mAbs in 96-well flat bottom Greiner Microtiter plates, mAbs were diluted to the required concentrations in carbonate buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH9.6, filtered through a 40 μm cell strainer) and 50-100 μl was added per well. After overnight incubation at 4 °C, plates were washed 3 times with 200 μl PBS, and blotted dry after the final wash. Plates were incubated with 250 μl RPMI regular medium for 30 minutes at 37 °C in 5 % CO_2 to block non-specific binding sites, then washed 3 times with 200 μl PBS prior to the addition of cells.

2.10.1.2. Stimulation of T cells

T cells were stimulated with an anti-CD3 mAb with or without CD81 cross-linking, and T cell activation was assessed by analysis of activation marker expression or measurement of IFN-γ production.

Greiner microtitre plates were coated with a range of concentrations of anti-CD3 mAb (0-1 μg/ml) with or without anti-CD81 mAb (clone JS-81 or 1.3.3.22) or IgG1 (0-10 μg/ml). T cells were purified by negative selection as described in section 2.2.4. In some experiments, purified T cells were incubated with IgG1 at 5 μg/ml per 5 x 10^6 cells in RPMI regular medium for 30 minutes at 4 °C prior to addition to plates, to block Fc receptors. Fc-blocked cells were washed once in 50 ml RPMI regular medium prior to use. Cells were stimulated overnight in experiments reading out up-regulation of expression of activation markers or for 2 days in experiments reading out IFN-γ production at 37 °C in 5 % CO_2 at a density of 2-5 x 10^5 cells in 200 μl RPMI regular medium per well. Cell supernatants were stored at -20 °C prior to measurement of their IFN-γ content.

2.10.1.3. Stimulation of NK cells

NK cells were stimulated with mAbs against activating NK cell receptors (CD16, NKG2C, NKG2D, CD161) or cytokines with or without CD81 cross-linking, and NK cell activation was assessed by analysis of activation marker expression or measurement of IFN-γ production.

In experiments where NK cells were stimulated by CD16 ligation, Greiner microtitre plates were coated with a range of concentrations of anti-CD16 mAb (0-10 μg/ml) with or without anti-CD81 mAb (clone JS-81 or 1.3.3.22) or IgG1 (0-10 μg/ml). NK cells

were purified from PBMCs by negative selection as described in section 2.2.4. In some experiments, PBMCs or purified NK cells were incubated with 5 μg/ml of IgG1 per 5 x 10^6 cells in RPMI regular medium for 30 minutes at 4 °C prior to addition to plates, to block Fc receptors. After washing, cells were added to plates at 5 x 10^5 (PBMCs) or 2 x 10^5 (purified NK cells) cells per well in 200 μl RPMI regular medium and incubated overnight or for 2 days at 37 °C in 5 % CO_2.

In experiments where NK cells were stimulated using mAbs against other NK activation molecules, Greiner microtitre plates were coated with anti-NKG2C mAb, anti-NKG2D mAb, isotype-matched control mAbs or a recombinant human MICA/Fc chimera protein or control human IgG1/Fc protein, at 5 or 10 μg/ml (or other amounts as indicated) with or without anti-CD81 mAb (clone JS-81) or IgG1 at 10 μg/ml. PBMCs were added to the plates at 5 x 10^5 cells per well in 200 μl RPMI regular medium with or without human recombinant IL-2 at 2 U/ml and incubated for 3 days at 37 °C in 5 % CO_2.

In experiments where NK cells were stimulated by cytokines, Greiner microtitre plates were coated with anti-CD81 mAb (clone JS-81) or IgG1 at 10 μg/ml. Cells were added at 5 x 10^5 PBMCs per well in 200 μl RPMI regular medium with or without the following cytokines: (1) 0-25 U/ml IL-2, (2) 0-1.25 μg/ml IL-12, (3) 0-0.25 μg/ml IL-18, and (4) 0-2 ng/ml IL-15. The plates were then incubated overnight at 37 °C in 5 % CO_2. Cell supernatants were stored at -20 °C prior to measurement of their IFN-γ content.

2.10.1.4. Stimulation of monocyte-derived DCs

Monocyte-derived DCs were stimulated with LPS, polyriboinosinic:polyribocytidylic acid poly(I:C) or a mAb against CD40 with or without CD81 cross-linking, and their activation was assessed by analysis of activation marker expression or measurement of supernatant cytokine content.

Greiner microtitre plates were coated with anti-CD81 mAb (clone JS-81) or IgG1 at a concentration of 5 or 10 μg/ml. In experiments where monocyte-derived DCs were stimulated with an anti-CD40 mAb, plates were coated with a range of concentrations of anti-CD40 mAb (0-100 μg/ml) with or without anti-CD81 mAb or IgG1. In experiments where DCs were stimulated with LPS, cells were washed in 50 ml RPMI regular medium 3 times to get rid of the polymyxin B from their previous culture medium and kept in DC culture medium without polymyxin B for the remainder of the experiment. Fc-receptors on monocyte-derived DCs were blocked by incubation of the cells with 10 μg/ml of anti-CD32 mAb per 5 x 10^6 cells in DC culture medium without polymyxin B (LPS stimulation) or with polymyxin B (for stimuli other than LPS) for 30 minutes at 4 °C. After washing, 2-5 x 10^5 cells were added per well in 200 μl of appropriate media. In experiments where cells were stimulated with LPS or poly(I:C), various concentrations of LPS (0-300 ng/ml) or poly(I:C) (0-25 μg/ml) were added to the medium. Cells were incubated at 37 °C in 5 % CO_2 overnight in the case of LPS stimulation or for 48 hours in the case of anti-CD40 mAb or poly(I:C) stimulation. Supernatants were stored at -20 °C prior to measurement of their cytokine content.

2.10.2. Analysis of cell surface marker on or intracellular protein expression by stimulated cells

Activation of cells was determined by staining with mAbs against appropriate cell surface markers or intracellular proteins. Surface staining was carried out as described in section 2.3; the concentrations of each mAb used are indicated in section 2.1.5. Staining was assessed by flow cytometry using a FACSCalibur™ flow cytometer and analysed using CellQuest™ software.

The activation marker used for assessing T/NK cell stimulation was CD69. Purified T cells were co-stained with PE-Cy5-conjugated anti-CD4, APC-conjugated anti-CD8, and FITC-conjugated anti-CD69 mAbs. In order to detect CD69 on NK cells, PBMCs or purified NK cells were co-stained with PE-conjugated anti-CD3 mAb, 5PC-conjugated anti-CD56 mAb and FITC-conjugated anti-CD69 mAb. In some experiments, intracellular perforin staining of NK cells was carried out. Here, cells were stained with mAbs against the surface markers CD3 and CD56, then cells were transferred into a FACS tube and washed with 2 ml FACS buffer. 200 µl Perm solution (Cytofix/Cytoperm kit) was added per tube and the cells were incubated for 20 minutes at 4 °C. After the permeabilisation step, the cells were washed once in 2 ml 1x Perm wash (Cytofix/Cytoperm kit). Similar washes were performed between all subsequent steps. Cells were then incubated with 2.5 µl FITC-conjugated anti-perforin mAb or FITC-conjugated isotype control for 30 minutes at 4 °C. NK cell staining was assessed using a FACSCalibur™.

The activation of monocytes was assessed by staining with mAbs to CD86 and HLA-DR. The maturation or activation of MDDCs was assessed by staining with mAbs specific to CD40, CD54, CD80, CD83, CD86, CD95, HLA-A,B,C, HLA-DR, CCR5 and CCR7. The mean fluorescence intensity (MFI) of surface marker staining was

calculated as the MFI of cells stained with marker-specific mAb minus the MFI of cells stained with an isotype-matched control mAb.

2.10.3. Detection of cytokines by ELISA

The IL-12 p40 levels present in culture supernatants harvested from stimulated monocyte-derived DCs and IFN-γ levels present in supernatants from stimulated PBMCs/purified NK cells were assessed by Enzyme-Linked ImmunoSorbent Assay (ELISA), using human IL-12 p40 or IFN-γ ELISA kits, following the manufacturer's instructions. ELISA Maxisorp plates were coated with 100 µl/well of the capture mouse anti-human IL-12 p40 mAb or anti-human IFN-γ mAb at 4 µg/ml in PBS. The plates were sealed and incubated overnight at room temperature. The plates were washed three times in 300 µl washing buffer [0.05 % (v/v) Tween-20 in PBS] per well between all subsequent steps. The plates were blocked by addition of 300 µl of blocking buffer [1 % (w/v) BSA, 5 % (w/v) sucrose in PBS] per well for 1-2 hours at room temperature. Dilutions of the standard and samples were prepared in 100 µl/well of reagent diluent [0.1 % (w/v) BSA, 0.05 % (v/v) Tween-20 in TBS (150 mM NaCl, 20 mM Trizma base in H_2O) at pH 7.2-7.4, filtered through 40 µm cell strainer]. The standard consisted of recombinant human IL-12 p40 or human IFN-γ and two-fold dilutions were made starting from a concentration of 2000 pg/ml IL-12 p40 or 1000 pg/ml IFN-γ. Test samples were thawed just before adding to the wells. They were added neat (100 µl) and at a series of two-fold dilutions. All dilutions of the standard and samples were tested in duplicate. The plates were incubated for 2 hours at room temperature. IL-12 was then detected by addition of 100 µl/well of a biotinylated goat anti-human IL-12 p40 detection mAb at 175 ng/ml in reagent diluent and IFN-γ was detected by addition of 100 µl/well of a biotinylated goat anti-human IFN-γ detection mAb at 100 ng/ml. These

reagents were incubated on the plates for 2 hours at room temperature. The biotinylated mAbs were detected using 100 µl/well of HRP-conjugated streptavidin diluted 1:200 in reagent diluent, which was incubated on the plates for 20 minutes at room temperature in dark. HRP activity was measured using 100 µl TMB substrate solution (described in section 2.5.2) per well. Colour development was allowed to occur for 20 minutes at room temperature in dark. The reaction was stopped by addition of 50 µl per well of 10 % sulphuric acid. Absorbance values were determined at 450 nm using a SpectraMax 340 reader. Data was analysed with Softmax Pro software (Molecular Devices Corp, Winnersh, UK).

2.10.4. Detection of cytokines by CBA assay

A variety of cytokines in the culture supernatants were measured using BD Cytometric Bead Array (CBA) kits, following the manufacturer's instructions. A human inflammation CBA kit was used to measure IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12 p70 protein levels in supernatants from human monocyte-derived DCs. In CBA assays, capture beads which have discrete fluorescence intensities bind to specific cytokines and these cytokines are further detected using PE-conjugated mAbs specific to the cytokines.

Test supernatants were thawed just before starting the assay. Serial dilutions of the standards were prepared in assay diluent to cover concentrations from 20-5000 pg/ml of each cytokine being detected. Master-mixes of the capture beads were prepared by mixing 6 µl of each cytokine capture bead per sample to be tested. 35 µl of supernatant samples or the dilutions of the standards were mixed with 35 µl of the capture bead master-mix and 35 µl of detection reagent, which contained PE-conjugated anti-human/mouse cytokine mAbs. They were incubated for 3 hours at room temperature in

the dark. Samples were then washed in 1 ml of wash buffer and centrifuged at 1400 rpm for 5 minutes in a Sorvall RT7 centrifuge. The supernatant was carefully discarded and the cytokine levels were assessed using a FACSCalibur™ flow cytometer with FACSComp software (Beckton Dickinson) for the instrument settings. The data was analysed using CellQuest™ software.

2.10.5. NK cell cytotoxicity assay

The cytolytic activity of NK cells was determined using standard ^{51}Chromium (Cr) release assays (Friberg *et al.*, 1996), using K562 cells as targets.

K562 cells were maintained in RPMI regular medium and subcultured twice a week. To label the target cells with chromium, they were pelleted in a 15 ml Falcon tube, and 100 μCi radioactive sodium chromate ($Na^{51}CrO_4$) was added per 10^6 K562 cells. The cells were incubated for 1 hour at 37 °C in 5 % CO_2, then were washed 4 times in 5 ml phenol-red free RPMI [phenol-red free RPMI supplemented with 10% FCS (Invitrogen)]. The labelled target cells were resuspended in phenol-red free RPMI at 1.5 x 10^5 cells/ml, so that 1.5 x 10^4 cells could be added to assay wells in a 100 μl volume.

Greiner 96 well plates were prepared by coating wells with anti-CD81 mAb (clone JS-81) or IgG1 at 10 μg/ml, and blocked in RPMI regular medium as described in section 2.10.1.1. NK cells were purified from PBMCs by negative selection as described in the section 2.2.4. Purified NK cells were resuspended in phenol-red free RPMI at concentrations between 3 x 10^6 - 3 x 10^5 cells/ml, so that 100 μl could be added per assay well to give effector (E): target (T) ratios of 20:1, 10:1, 5:1, and 2:1, respectively. NK cells were added to the antibody-coated 96-well plates and incubated for at least 30

minutes at 37 °C in 5 % CO_2 before the target cells were added. 100 µl of labelled target cells were then added to all wells which contained effector cells. To obtain a measurement of the spontaneous release of ^{51}Cr from the target cells, wells containing target cells plus 100 µl phenol-red free RPMI were also set up. To determine the maximal release of ^{51}Cr from the target cells, further wells containing target cells plus 100 µl of 5 % (v/v) Triton X-100 were also set up. All variables were assayed in triplicate.

Plates were incubated for 5 hours at 37 °C in 5 % CO_2. 40 µl of supernatant was then harvested from each well and transferred to a counting plate which contained 100 µl of Optiphase scintillant per well. The plate was sealed and left overnight at room temperature to allow homogenous mixing of supernatant and scintillant. ^{51}Cr was then measured using a Trilux 1450 Microbeta counter (Wallac, Turku, Finland).

Results from cytolysis assays are reported as the percentage specific lysis at each E:T ratio. The percentage of specific lysis was calculated using the following equation:

$$\% \text{ specific lysis} = \frac{\text{Mean experimental release} - \text{Mean spontaneous release}}{\text{Mean maximum release} - \text{Mean spontaneous release}} \times 100$$

2.11. CD81 KO mice

2.11.1. Screening for CD81 KO mice

Heterozygous CD81 KO mice (Tsitsikov *et al.*, 1997) were obtained from Dr Raif Geha (Harvard Medical School, Boston, USA) and were re-derived by Charles River Laboratories (Margate, Kent, UK), a process that involved breeding the heterozygous CD81 KO mice to normal C57BL/6 (B6) females, delivering the offspring by Caesarian

section under sterile conditions, and fostering onto SPF mothers. After re-derivation the mice were maintained in the specific pathogen free (SPF) breeding unit at the IAH, UK. The mice received were screened to identify animals carrying the CD81 KO mutation. CD81 heterozygous mice were then bred together, and the offspring were screened to identify heterozygous animals (which were used for further breeding) and mice homozygous for the CD81 mutation and wild-type animals (which were used for experimental purposes).

All animal studies were carried out in accordance with UK Home Office regulations, and were approved by the site ethical review committee.

PCR was used to distinguish homozygous/heterozygous CD81 KO mice from wild-type mice. DNA was obtained from mouse tail biopsies using a DNeasy tissue kit, and quantified using a GeneQuant Pro UV spectrophotometer. DNA samples were then tested by PCR, using a set of primers designed to amplify a 328 base pair (bp) fragment from the neomycin resistance gene (present in heterozygous and homozygous CD81 KO mice): 5-ATCGGCCATTGAACAAGATGGA-3' and 5'-AGCAAGGTGAGATGACAGGAG-3'. The primers were designed, keeping the primer length to 20-23 bp, GC content to 40-60 %, and ensuring that the third bp from the 3' end was a G or C residues. PCR reactions were set up as follows: 0.1 μg DNA, 10 pmol each primer, 1 x super TAQ PCR buffer, 500 μM each deoxyribonucleotide triphosphate (dNTP, 2 mM final concentration), 1 mM $MgCl_2$ and 0.85 U Taq polymerase, made up to 25 μl in H_2O. A negative control without DNA was also included. The PCR conditions involved a 5 minute hot start at 94°C followed by 30 cycles of 95 °C for 30 seconds, 56.1 °C for 30 seconds and 72 °C for 1 minute, with a final extension of 5 minutes at 72 °C in the last cycle in GeneAmp PCR system 9700

(Applied biosystems). The amplified products were run on a 1 % agarose gel together with a 250 bp DNA ladder. DNA bands were visualised under UV light and photographed with a Polaroid GelCam (Jencons, Forest Row, UK).

To distinguish heterozygous CD81 KO mice from homozygous CD81 KO mice, surface expression of CD81 on peripheral blood lymphocytes (PBL) was checked by mAb staining and FACS analysis. Blood was collected from a tail vein into a 1.5 ml Eppendorf tube containing 80 µl heparin at 100 IU/ml. The blood was diluted 1:2 by adding 200 µl PBS, and was mixed well by votexing. 450 µl pre-warmed Histopaque-1083 was underlaid and the samples were centrifuged at 1600 rpm at room temperature for 20 minutes in a Sorvall RT7 centrifuge with the brake off. PBL were harvested from the interface and washed in a FACS tube with 2 ml of HBSS solution [1 x Hanks Balanced Salt Solution, 2 % (v/v) FCS, 0.2 % (v/v) sodium azide in H_2O].
5 µl PE-conjugated anti-mouse CD81 mAb was added to the cells in the residual HBSS solution. An aliquot of cells from some of the samples was stained with 5 µl PE-conjugated hamster IgG1 isotype control instead. The cells were incubated with the mAbs for 30 minutes at 4 °C, then washed once in 2 ml HBSS solution. Surface CD81 expression was detected using a FACSCalibur™ flow cytometer and analysed using CellQuest™ software.

2.11.2. Production and stimulation of murine bone marrow derived-DCs

Hind limbs were collected from three CD81 KO mice and three wild-type control animals, and femurs and tibia were dissected using forceps and scissors. After removing the flesh, the bones were cleaned by spraying with 70 % alcohol and placed in complete DC medium [RPMI supplemented with 10 % (v/v) FCS, 1ug/ml penicillin and

streptomycin, 100 U/ml polymyxin B, and 50 mM β-mercaptoethanol]. The ends of the bones were then cut off to create a hollow tube. Using a 27G needle attached to a syringe containing complete DC medium, the marrow was flushed out of the bones so that these became completely white. The resulting cell suspension was collected into a 50 ml Falcon tube and the cells were counted, including only large irregular cells. The cells were adjusted to 2 x 10^5 cells per ml in complete DC medium supplemented with 20 ng/ml mouse GM-CSF. They were then plated into 100 x 150 mm Petri dishes at 10 ml per dish and incubated at 37 °C in 5 % CO_2 for 8 days to allow bone marrow precursors to differentiate into DCs. At day 3 of culture, the dishes were softly swirled and 10 ml fresh complete DC medium supplemented with 20 ng/ml mouse GM-CSF was added per dish. At day 6, the dishes were swirled again and 10 ml old medium removed from each dish. The medium collected was centrifuged to rescue the cells therein, and the cell pellet was resuspended in 10 ml fresh complete DC medium supplemented with 20 ng/ml mouse GM-CSF. The fresh medium and cells were then added back to the dish for further incubation.

The process of differentiation from bone marrow precursors into bone marrow-derived DCs (BMDCs) was monitored by counting the cells and analysing their expression of CD11c at days 3, 6 and 8 of culture. BMDCs were harvested from one dish, counted and 1 x 10^6 cells were stained with anti-mouse CD11c mAb. The method for surface staining of cells was described in section 2.3.

Cells were harvested at day 8 of culture and counted. In experiments where BMDCs were stimulated with LPS, BMDCs were washed 3 times in 50 ml RPMI regular

medium to get rid of the polymyxin B from the culture medium, and were then kept in complete DC medium without polymyxin B for the rest of the steps.

Cells were resuspended to 2.5 x 10^6/ml in complete DC medium with [poly(I:C) or anti-CD40 mAb stimulation] or without polymyxin B [LPS stimulation], and were plated into 6-well plates at 2 ml/well. BMDCs were stimulated with a range of concentrations of LPS (0-300 ng/ml), poly(I:C) (0-100 μg/ml) or anti-mouse CD40 mAb or IgM isotype control (0-10 μg/ml). The cells were incubated for 24 hours at 37 °C in 5 % CO_2. The cells were then harvested for phenotypic analysis and supernatants were stored at -20 °C prior to measurement of their cytokine content.

2.11.3. Isolation of spleen DCs

Spleens were collected from four CD81 KO mice and four wild-type B6 mice; each set of spleens was kept in 10 ml PBS. After removing any extra fat around the spleens, 200 μl of enzyme mix [1 mg/ml collagenease, 0.5 mg/ml DNase, 2 % FCS (v/v), 1 μg/ml penicillin and streptomycin in RPMI] was injected into each spleen. The spleens were kept in a 50 ml Falcon tube and incubated in a water bath at 37 °C for 15 minutes. The digested spleens were then disrupted by passing through a 40 μm cell strainer. The cells were washed in RPMI regular medium and counted. An aliquot of cells was removed at this stage for surface CD11c staining to enable the percentage of DCs in the total splenocyte population to be determined.

The remaining cells were resuspended in 3 ml Nycoprep 1.077A and mononuclear cells were separated using Nycoprep density gradient centrifugation. The cell suspension was layered onto 3 ml fresh Nycoprep in a 15 ml Falcon tube. 2 ml of FCS-EDTA (0.01M EDTA in FCS) was added on top of cell suspension. The cells were centrifuged at 3000 rpm for 20 minutes at 4 °C in Sorvall RT7 centrifuge. Several fractions were created

after the Nycoprep density gradient separation. All fractions from the top layer to the second interface from the bottom were collected, resuspended in RPMI regular medium and the number of cells obtained was determined.

$CD11c^+$ cells (spleen DCs) were then isolated by positive selection using MACS CD11c MicroBeads, using the method described in section 2.2.4. The MACS buffer was supplemented with 100 U/ml polymyxin B.

2.11.4. Analysis of cell surface marker expression on DCs

The activation and maturation of BMDCs or spleen DCs was determined by staining for cell surface activation markers. The method for the surface staining was as described in section 2.3. Cells were stained with biotin-conjugated mAbs against mouse CD86, CD80, CD40, CD54, H-2Kb, or 1A/1E. BMDCs were co-stained with a FITC-conjugated anti-CD11c mAb. Spleen DCs were co-stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD11c mAbs to identify $CD4^+$ DCs, FITC-conjugated anti-CD8 and PE-conjugated anti-CD11c mAbs to identify $CD8^+$ DCs, or alternatively FITC-conjugated anti-CD49b/Pan NK and PE-conjugated anti-CD45R/B220 mAbs to enable identification of plasmacytoid DCs ($CD49b^-$ $B220^+$). Biotin-conjugated mAbs were detected using APC-conjugated streptavidin. The concentrations of mAbs used for staining are indicated in section 2.1.6. Staining was assessed using a FACSCalibur™ flow cytometer and analysed using CellQuest™ software.

2.11.5. Detection of murine cytokines by ELISA or CBA

IL-12 p70 levels present in culture supernatants harvested from stimulated BMDCs were assessed by ELISA, using a murine IL-12 p70 ELISA kit, following the manufacturer's instructions. The method was described in section 2.10.3.

A variety of cytokines in the culture supernatants were measured using BD CBA kits, following the manufacturer's instructions. A mouse inflammation CBA kit was used to measure IL-6, IL-10, MCP-1, IFN-γ, TNF-α and IL-12 p70 protein levels in supernatants from stimulated BMDCs. The method was described in section 2.10.4.

2.12. Statistical analysis

The statistical significance of differences in the level of CD81 expression on different PBMC subsets was addressed by analysis of variance (ANOVA), using a general linear model. The significance of differences in the % of T cells or NK cells expressing CD69 under different experimental conditions was also addressed by ANOVA, using a general linear model with three-way interaction. All statistical analyses were done using Minitab 1.4 software (Minitab Ltd., Coventry, UK).

Chapter 3 Binding of E2 proteins to different PBMC subsets

3.1. Introduction

As reviewed in the main introduction, HCV virions are enveloped and are thought to express heterodimeric complexes of the two viral glycoproteins, E1 and E2, on their surface. The HCV E2 glycoprotein has been shown to bind to several different cell surface receptors, including CD81 (Pileri *et al.*, 1998), SR-BI (Scarselli *et al.*, 2002), the LDL receptor (Agnello *et al.*, 1999; Wunschmann *et al.*, 2000), DC-SIGN and L-SIGN or DC-SIGNR (Gardner *et al.*, 2003; Lozach *et al.*, 2003; Pohlmann *et al.*, 2003) and GAGs (Basu *et al.*, 2004; Takikawa *et al.*, 2000; Yagnik *et al.*, 2000). Interactions between virion glycoproteins and host cell receptors are required for virion attachment and entry into host cells, and are among the factors that determine viral tropism. It has also been shown that virion glycoprotein-host cell receptor interactions can modulate host cell functions. Notably, cross-linking of CD81 on T cells by E2 was found to co-stimulate their activation in response to signalling via CD3 (Wack *et al.*, 2001).

There are a number of studies supporting HCV infection of haematopoietic cells *in vivo* (Bouffard *et al.*, 1992; Goutagny *et al.*, 2003; Lerat *et al.*, 1998; Muller *et al.*, 1993; Zignego *et al.*, 1992). However, although the ability of E2 proteins to bind to lymphocyte cell lines such as Daudi (B cell line) and Molt-4 (T cell line) cells has been reported (Flint *et al.*, 1999b; Roccasecca *et al.*, 2003; Wellnitz *et al.*, 2002), the interaction of E2 proteins with *ex vivo* PBMCs has not been examined in depth.

The objective of the work described in this chapter was to characterise the expression of HCV (co)-receptors CD81 and SR-BI on PBMC subsets and to analyse the binding of HCV E2 glycoprotein to CD81 and/or other host cell surface receptors on PBMC, to give insight into how different cell subpopulations may become infected with HCV *in vivo*, and also into the potential for E2 to modulate the functions of different immune system cell types via interaction with CD81.

As cell culture systems that supports HCV replication have only recently begun to be developed, recombinant viral envelope proteins are typically used to study ligand-receptor interactions. In this project, four different forms of HCV glycoproteins were used: (1) soluble recombinant $E2_{660/661}$ proteins produced by transfection of eukaryotic cells with plasmids encoding truncated E2 genes; (2) VLPs expressing heterodimers of E1 and E2 that were produced in a baculovirus expression system; (3) retroviral particles pseudotyped with the HCV glycoproteins (heterodimers of E1 and E2); and (4) HCV virions that were purified from the serum of a patient infected with HCV.

Soluble recombinant $E2_{660/661}$ proteins produced by transfection of eukaryotic cells with plasmids expressing truncated E2 genes have been well characterised. The presence of an ER retention signal within E2 makes it difficult to purify full-length recombinant E2 and distinguish properly folded molecules from non-productive molecules. Truncation of E2 to remove the hydrophobic transmembrane domain and C-terminal region results in secretion of E2 (Flint *et al.*, 1999b; Matsuura *et al.*, 1994; Michalak *et al.*, 1997). It was shown that truncation at position 660 or 661 increased secretion of E2 and recognition by conformational dependent mAbs compared to truncation at aa 688, 704 or 715 (Cocquerel *et al.*, 1998; Flint *et al.*, 1999b; Heile *et al.*, 2000; Michalak *et al.*, 1997). $E2_{660/661}$ preparations were found to containe minimal amounts of disulfide bridged aggregates and to be folded in a manner comparable to E2 in E1E2 complexes (Flint *et al.*, 1999a; Michalak *et al.*, 1997). $E2_{660/661}$ is thus useful soluble form of E2, which is predicted to mimic the native form of E2.

$E2_{660/661}$ is present in both the supernatant and cell-associated fractions of transfected cells; but the supernatant and cell-associated E2s are differently glycosylated.

Glycosylation is known to modulate protein-protein interaction (Gahmberg & Tolvanen, 1996). Although deglycosylation of cell-associated $E2_{715}$ did not affect its cell binding ability (Flint & McKeating, 2000), cell-associated $E2_{661}$ reportedly had a stronger affinity for human CD81 than supernatant $E2_{661}$, and was recognised better by sera from chronically infected patients (Flint *et al.*, 2000; Heile *et al.*, 2000). It is thus speculated that cell-associated $E2_{661}$ may mimic native E2 more closely than supernatant $E2_{661}$. This is perhaps not unexpected, as HCV virions are thought to mature by budding from the ER (Bartenschlager & Lohmann, 2000; Dubuisson *et al.*, 2002). In this study, it was initially planned to use both supernatant and cell-associated truncated E2 proteins.

When studying the functions of E2, it is important to consider that differences exist between soluble E2 proteins and the native viral form. Notably, the latter is found as heterodimers with E1, and is expressed in an array on the virion surface. Several surrogate models of HCV virions have thus been developed to investigate the interaction of HCV with host cells. The results obtained using these surrogate models may be more representative of the native activity of E2.

The baculovirus-insect cell expression system has been successfully used to produce HCV VLPs (Baumert *et al.*, 1998; Clayton *et al.*, 2002; Steinmann *et al.*, 2004; Triyatni *et al.*, 2002a; Triyatni *et al.*, 2002b). Advantages of using the baculovirus expression system are: (1) that eukaryotic insect cells have a number of co- or posttranslational modifications similar to mammalian cells; and (2) that this system allows high level expression of heterologous proteins (Baumert *et al.*, 1998). When the HCV structural proteins are co-expressed in the baculovirus expression system, they are assembled into enveloped VLPs with morphological, biophysical and antigenic properties similar to

those of native virions isolated from HCV-infected patients (Baumert *et al.*, 1998; Clayton *et al.*, 2002; Triyatni *et al.*, 2002a).

As an alternative surrogate model of HCV virions, several groups have successfully developed retroviral particles pseudotyped with functional HCV E1E2 glycoproteins (Bartosch *et al.*, 2003a; McKeating *et al.*, 2004). The E1E2 proteins on these particles have a mature conformation similar to that on native HCV particles (Op De Beeck *et al.*, 2004). The advantage of using pseudotyped retroviral particles is that they are highly infectious, which allows both viral binding and entry to be studied. Retroviruses are particularly useful platforms compared with other pseudotyped viruses, because their core proteins can incorporate variety of different cellular and viral glycoproteins (Sandrin *et al.*, 2002; Sung & Lai, 2002), and they can easily package and transfer genetic markers, such as GFP and luciferase genes, into the DNA of infected cells (Bartosch *et al.*, 2003a). Using these genetic markers, it is possible to determine which cell types HCV glycoproteins can interact with and gain entry into.

In addition to these surrogate models, native HCV virions purified from serum of a patient infected with HCV were also used in this project. These provide the ideal reagent for analysis of HCV interaction with host cell receptors, since any forms of recombinant protein could be structurally different from the native form, and this may affect interactions with putative receptors. However the use of virions is hampered by limitations on their availability. Due to the difficulties in purifying large quantities of HCV virions from patients, only a limited number of studies have been done so far using native HCV virions (Agnello *et al.*, 1999; Hamaia *et al.*, 2001; Wunschmann *et al.*, 2000).

In this chapter, I thus analysed the expression of the putative HCV (co)-receptors CD81 and SR-BI on haematopoietic cell subsets, then addressed the binding of soluble E2 proteins and HCV VLPs, HCV pseudotyped retroviral particles and HCV virions to these cells. As detailed later in the chapter, all the HCV glycoproteins used were derived from genotype 1 viruses. Much work was done with (E1)E2 glycoproteins derived from the H77c strain of HCV, a cDNA clone of which was shown to be infectious in the chimpanzee model (Yanagi *et al.*, 1997). However some comparative studies were also carried out with soluble E2 proteins derived from other genotype 1a viruses.

3.2. Results

3.2.1. Analysis of CD81 and SR-BI expression on PBMCs, monocyte-derived DCs and hepatocyte cell lines

It has been reported that the HCV E2 glycoprotein can bind to CD81 and SR-BI, and that both of these (co)-receptors are involved in HCV entry into hepatocytes (Bartosch *et al.*, 2003b; Zhang *et al.*, 2004). Although HCV is primarily a hepatotropic virus, certain PBMC subsets are also suggested to be infected with HCV *in vivo*. To gain insight into the relationship between expression of these putative (co)-receptors and the reported distribution of HCV RNA within immune system cell subsets *in vivo*, the expression of CD81 and SR-B1 on different PBMC subsets and monocyte-derived DCs was examined.

PBMCs from healthy donors were stained with fluorescent-conjugated mAbs specific for different surface markers to allow identification of PBMC subsets and simultaneous analysis of CD81 or SR-B1 expression on these cell types. NK cells were defined as $CD3^-CD56^+$ cells (Fig. 3.1-B). This population was subdivided into two subsets;

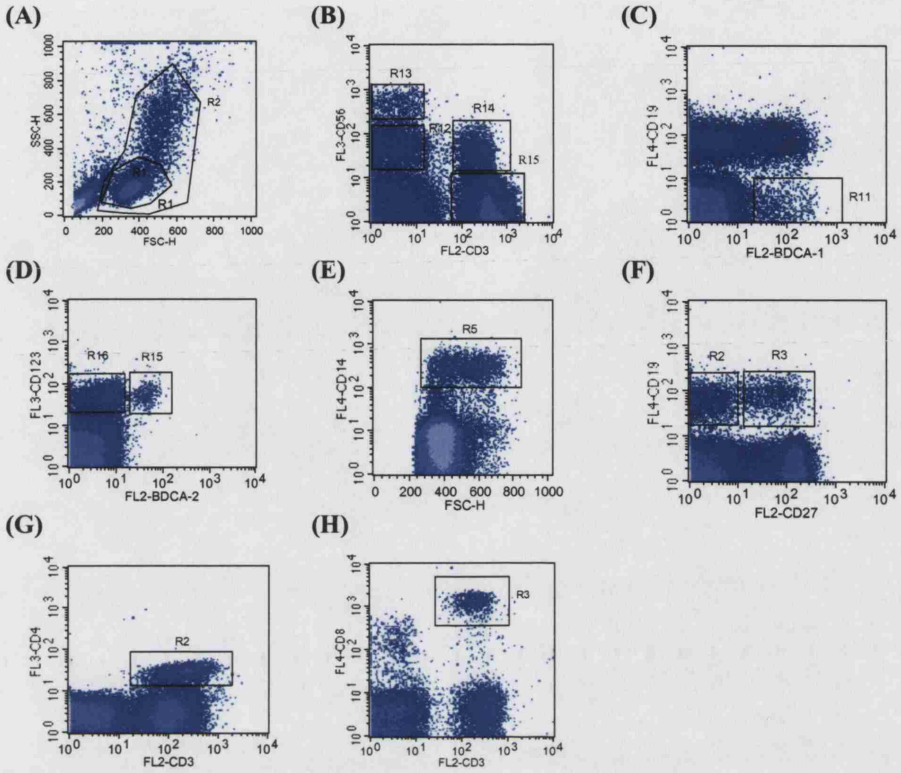


Figure 3.1. Identification of different PBMC subsets by FACS analysis.

PBMC subsets were identified by multi-colour flow cytometry, following staining with mAbs against differentially expressed surface markers. (A) Dotplot showing the forward scatter (FSC) vs side scatter (SSC) profile of a typical PBMC sample. The R1 gate was used for analysis of all PBMC subsets apart from monocytes. The R2 gate was used for analysis of $CD14^{high}$ cells. (B-H) Identification of different PBMC subsets. The PBMC subsets analysed and the phenotypic basis on which they were identified were as follows: $CD56^{dim}$ NK cells ($CD3^-$ $CD56^{dim}$, R12 in (B)), $CD56^{high}$ NK cells ($CD3^-$ $CD56^{high}$, R13 in (B)), natural T cells (NT, $CD3^+$ $CD56^+$, R14 in (B)), T cells ($CD3^+$ $CD56^-$, R15 in (B)), myeloid DCs ($CD19^-$ BDCA-1^+, R11 in (C)), plasmacytoid DCs ($CD123^+$ BDCA-2^+, R15 in (D)), granulocytes ($CD123^+$ BDCA-2^-, R16 in (D)), monocytes ($CD14^{high}$, R5 in (E)), naïve B cells ($CD19^+$ $CD27^-$, R2 in (F)), activated B cells ($CD19^+$ $CD27^+$, R3 in (F)), $CD4^+$ T cells ($CD3^+$ $CD4^+$, R2 in (G)), $CD8^+$ T cells ($CD3^+$ $CD8^+$, R3 in (H)). R2 in (G) and R3 in (H) were further subdivided into naïve or memory cells on the basis of CD45RA (naïve) or CD45RO (memory) expression (not shown).

$CD56^{high}$ NK (R13, Fig. 3.1-B) and $CD56^{dim}$ NK cells (R12, Fig. 3.1-B). NT cells were defined as $CD3^+$ $CD56^+$ cells (R14, Fig. 3.1-B). In some experiments, T cells were defined as $CD3^+CD56^-$ cells (R15, Fig. 3.1-B). Myeloid DCs were identified as being $CD19^-$ BDCA-1^+ (R11, Fig. 3.1-C). Plasmacytoid DCs were identified as $CD123^+$ BDCA-2^+ cells (R15, Fig. 3.1-D). The $CD123^+$ BDCA-2^- cell population (R16, Fig. 3.1-D) was also analysed; this is largely composed of granulocytes. Monocytes were identified as $CD14^{high}$ cells (R5, Fig. 3.1-E). B cells were identified as the $CD19^+$ population and subdivided into $CD27^-$ naïve B cells (R2, Fig. 3.1-F) and $CD27^+$ activated B cells / plasma cells (R3, Fig. 3.1-F). T cells were defined as $CD3^+$ $CD4^+$ T cells (R2, Fig. 3.1-G) or $CD3^+$ $CD8^+$ T cells (R3, Fig. 3.1-H). They were also stained with mAbs to CD45 isoforms to identify $CD45RA^+$ (naïve/memory) or $CD45RO^+$ (activated/memory) subsets. Individual populations were gated and then the percentage of cells expressing CD81 and the level of CD81 expression (which was reflected by the mean fluorescence intensity (MFI) of CD81-specific staining) was analysed as described in Fig. 3.2. Alternatively, SR-BI expression on different cell subsets was assessed.

The majority (>90 %) of cells in most PBMC subsets were found to express CD81 on their surface (Fig. 3.3-A). Exceptions were plasmacytoid DCs, only 58 % of which expressed detectable levels of CD81; and granulocytes, only 20 % of which expressed detectable levels of CD81. ANOVA was performed to determine whether there were statistically significant differences in the % of cells of different subsets expressing CD81. The % of granulocytes expressing CD81 was significantly lower than that of all other subsets except plasmacytoid DCs ($p<0.05$).

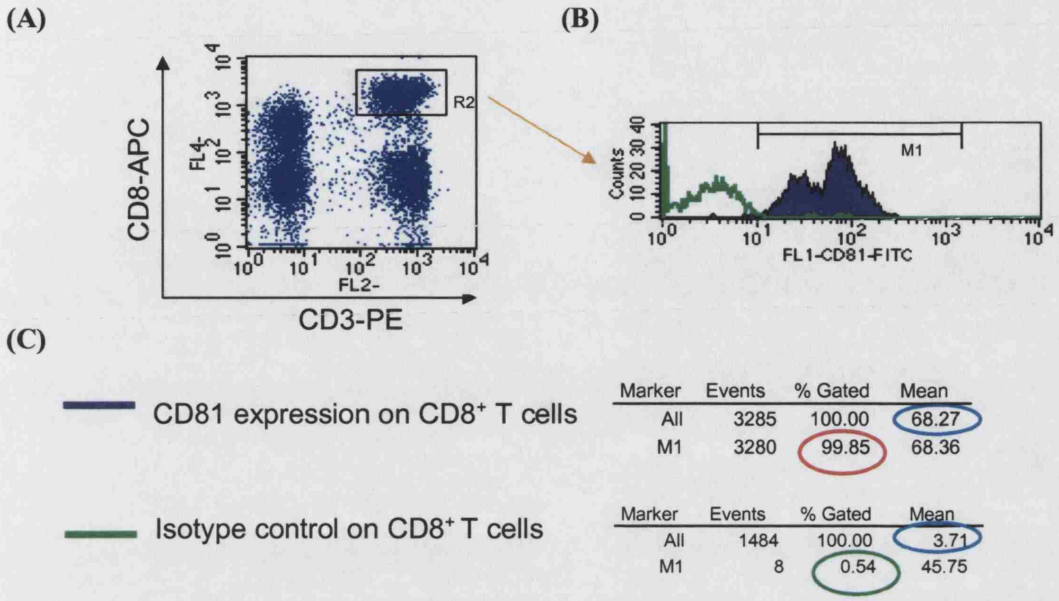
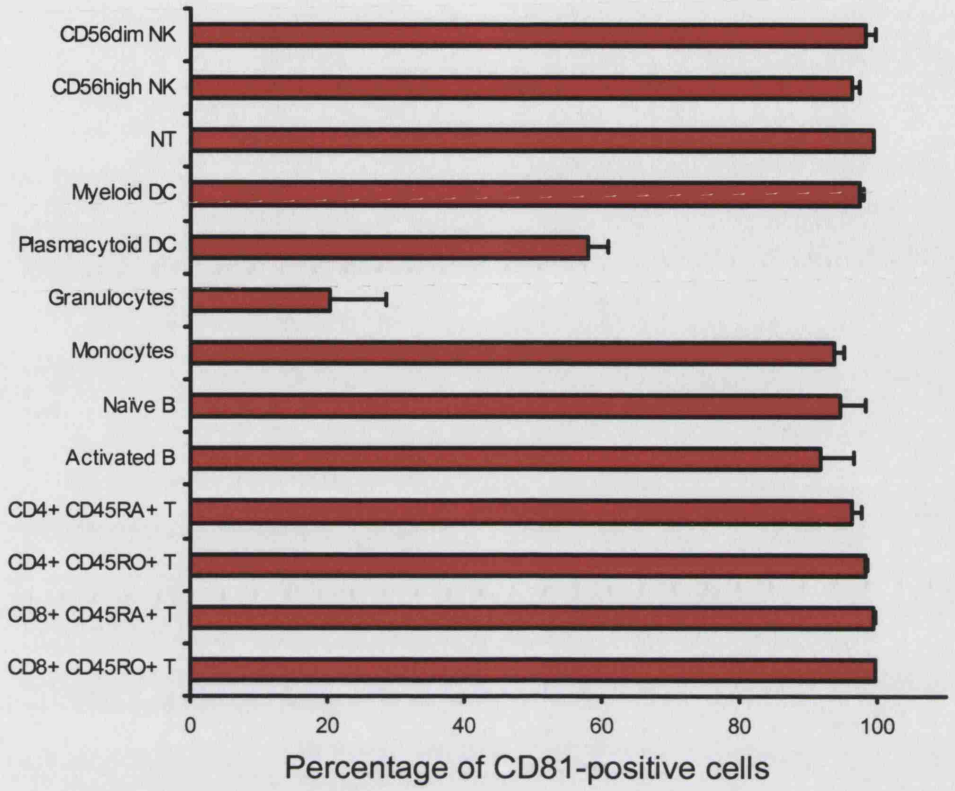


Figure 3.2. Analysis of CD81 expression on different PBMC subsets.

PBMCs were stained with a FITC-conjugated anti-CD81 mAb or isotype-matched (IgG1) control FITC-conjugated mouse mAb, together with combinations of fluorescent-conjugated mAbs allowing identification of different PBMC subsets as described in the legend to Figure 3.1. Various subsets were gated, and CD81 expression was investigated on the gated cells. As an example, the dotplot in (A) shows the CD3 versus CD8 staining profile of PBMCs and explains how CD81 expression on $CD3^+CD8^+$ T cells (R2) was analysed. In histogram (B), the purple shaded graph represents staining of $CD3^+CD8^+$ T cells with an anti-CD81 mAb, and the green line represents staining with an isotype-matched control mAb. The marker (M1) was set based on the isotype control, keeping the % of non-specifically-stained cells (green circle in (C)) lower than 2 %. The percentage of CD81 positive cells is indicated by the percentage of cells within the M1 marker (red circle in (C)). The mean fluorescence intensity (MFI) of cells stained with the CD81 or isotype control mAbs (blue circles) is also indicated in (C). The MFI of CD81-specific staining was calculated as the MFI of cells stained with the anti-CD81 mAb minus the MFI of cells stained with the isotype-matched control mAb (example is shown).

(A)


CD56dim NK
CD56high NK
NT
Myeloid DC
Plasmacytoid DC
Granulocytes
Monocytes
Naïve B
Activated B
CD4+ CD45RA+ T
CD4+ CD45RO+ T
CD8+ CD45RA+ T
CD8+ CD45RO+ T
0
20
40
60
80
100
Percentage of CD81-positive cells

(B)

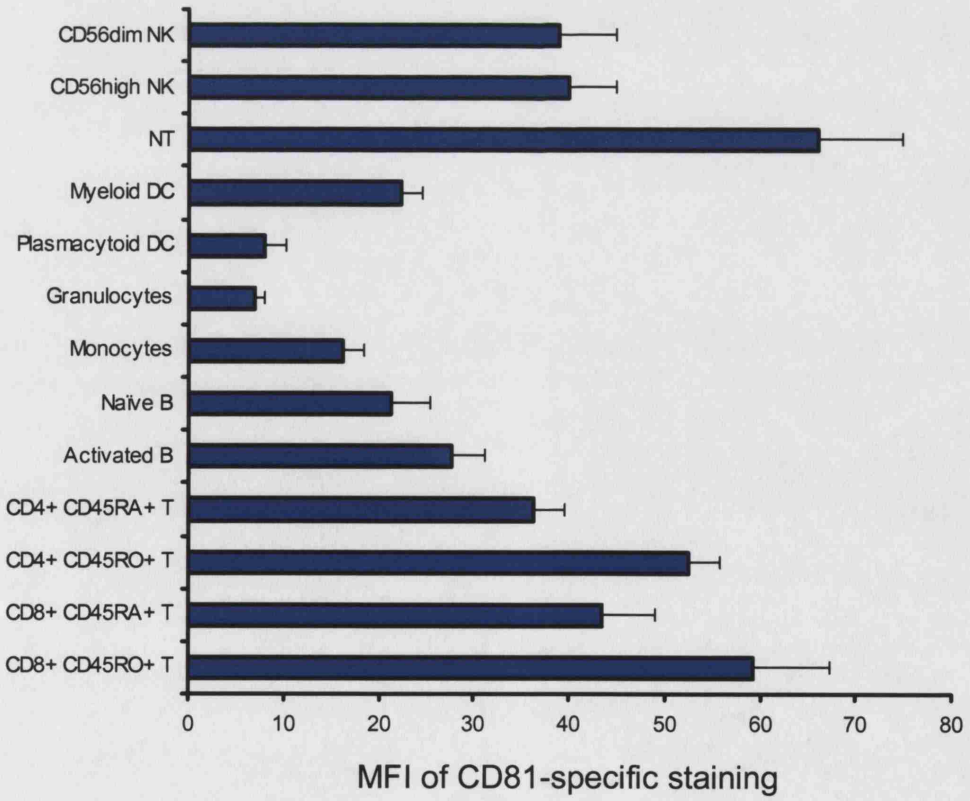

CD56dim NK
CD56high NK
NT
Myeloid DC
Plasmacytoid DC
Granulocytes
Monocytes
Naïve B
Activated B
CD4+ CD45RA+ T
CD4+ CD45RO+ T
CD8+ CD45RA+ T
CD8+ CD45RO+ T
0
10
20
30
40
50
60
70
80
MFI of CD81-specific staining

Figure 3.3. Expression of CD81 on PBMC subsets.

Surface expression of CD81 on PBMC subsets was analysed as described in Fig. 3.2. (A) Percentage of CD81-positive cells in different PBMC subsets. (B) Level of CD81 expression on different PBMC subsets. The data shown are the mean of results obtained when CD81 expression on PBMCs from three different donors was analysed in a single experiment. The error bars indicate one standard error above the mean. The data shown are representative of findings made in 2 independent experiments.

The level of CD81 expression on different cell subsets was very varied (Fig. 3.3-B). NK, NT and activated T cells expressed high levels of CD81. In particular, NT cells had the highest CD81 expression on the surface; the level was statistically higher than that on DCs, granulocytes, monocytes, B cells and naïve $CD4^+$ T cells ($p<0.05$). B cells, monocytes and myeloid DCs were found to express intermediate levels of CD81, whilst only low levels of CD81 were detected on plasmacytoid DCs and granulocytes. Activated/antigen-experienced $CD4^+/CD8^+$ T cells and B cells all expressed higher levels of CD81 than their naïve/more resting counterparts, although the differences were not statistically significant. Plasmacytoid DCs and granulocytes expressed statistically lower levels of CD81 than NK, NT and T cell subsets ($p<0.05$). It cannot be excluded that the variation observed in CD81 expression on different PBMC subsets may be due in part to CD81 being complexed with other cell surface proteins in such a way that the binding site of the anti-CD81 mAb used in this study is not readily accessible. The various levels of CD81 expression on each PBMC subset were determined using two anti-CD81 mAbs, clones JS-81 and 1.3.3.22, and both gave very similar results. However, it is likely that both mAbs recognise the same or closely located epitopes on CD81. Both mAbs are reported to bind to the LEL of CD81 and can block E2 binding to CD81 (Flint *et al.*, 1999b). These mAbs also compete to recognise CD81 on PBMCs (data not shown). However my results at least indicate that there is considerable variation in the level of E2-accessible CD81 expressed on different PBMC subsets.

SR-BI expression on different PBMC subsets was investigated using three different clones of anti-SR-BI mAb (clones 25, 3D5 and 6B8). Clone 25 had been generated using SR-BI aa 104 - 294 as an immunogen (PharMingen BD), whereas clones 3D5 and 6B8 were generated by immunisation of mice with a plasmid expressing full-length

human SR-BI (Bartosch *et al.*, 2003b). All three clones can recognise SR-BI on the cell surface, but only clone 3D5 is known to block the interaction between soluble E2 protein and SR-BI. SR-BI expression was found to be restricted to monocytes and DCs (Fig. 3.4). The majority of monocytes and plasmacytoid DCs expressed a moderate level of SR-BI, while myeloid DCs expressed a lower level of SR-BI. All the other PBMC subsets examined (NK cells, T cells, B cells and granulocytes) did not express detectable levels of SR-BI on their surface. Although all three anti-SR-BI mAbs could detect SR-BI expression on monocytes equally well, SR-BI expression on DCs was only detected using clones 3D5 and 6B8. Very similar results were obtained using clones 3D5 and 6B8. Even saturating concentrations of anti-SR-BI mAb clone 25 did not detect SR-BI on DCs. This may be due to the SR-BI epitope recognised by clone 25 being poorly accessible on DCs. It is thus possible that the failure to detect SR-BI expression on PBMC subsets other than monocytes and DCs may be due to differences in the SR-BI epitopes exposed on different cell subsets, rather than lack of SR-BI expression on most PBMC subsets.

Monocyte-derived DCs were produced by inducing the differentiation of peripheral blood monocytes into DCs in *in vitro* culture (see the Materials and Methods). Monocyte-derived DCs can be obtained in relatively large numbers, and are frequently used as a surrogate for *in vivo* DC populations. The majority of monocyte-derived DCs were found to express both CD81 and SR-BI (Fig. 3.5).

The expression of CD81 and SR-BI on different hepatocyte cell lines was also analysed. Huh-7, Hep16 and Hep3B cells were found to express CD81, but CD81 expression was not detected on HepG2 cells (Fig. 3.6). The level of CD81 expression on Huh-7 cells

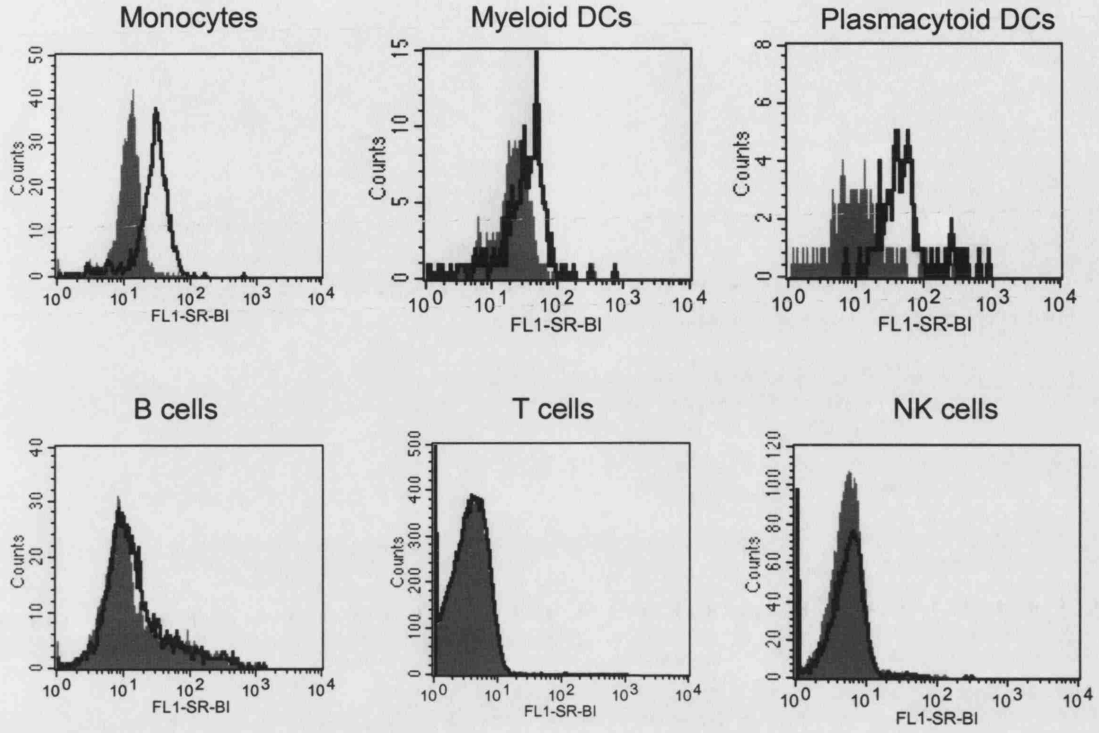


Figure 3.4. Expression of SR-B1 on PBMC subsets.

Surface expression of SR-BI on PBMC subsets was analysed by multi-colour flow cytometry. PBMCs were stained with an anti-SR-BI mAb (clone 3D5) or isotype-matched (IgG1) control mAb, and a FITC-conjugated secondary anti-mouse IgG Fab fragment, followed by combinations of fluorescent-conjugated mAbs allowing identification of different cell subsets, as described in the legend to Figure 3.1. Various cell subsets were gated, and SR-BI expression was investigated on the gated cells. In each panel, the grey shaded histogram represents staining of the indicated PBMC subpopulation with the isotype matched-control mAb and the thick black line represents staining with the anti-SR-BI mAb. The results shown are representative of findings made in 3 independent experiments using PBMCs from different donors.

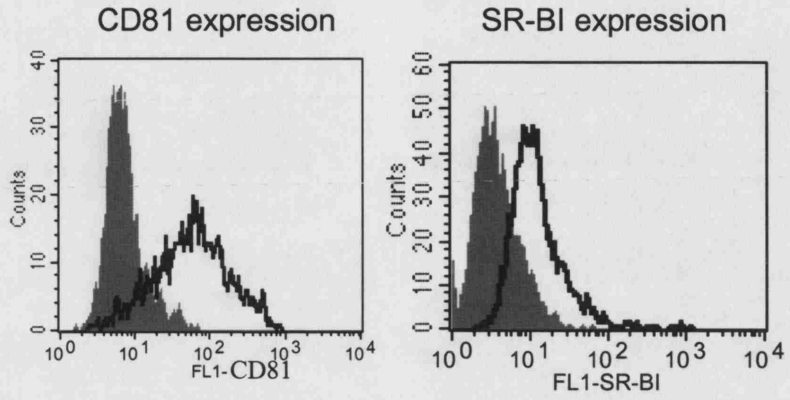


Figure 3.5. Expression of CD81 and SR-BI on monocyte-derived DCs.

Surface expression of CD81 and SR-BI on monocyte-derived DCs was analysed by multi-colour flow cytometry. Monocyte-derived DCs were produced as described in the Materials and Methods. In each panel, the grey shaded histogram represents staining of the monocyte-derived DCs with an isotype-matched control mAb and the thick black line represents staining with an anti-CD81 mAb (clone JS-81) or anti-SR-BI mAb (clone 3D5). The CD81 expression data are representative of findings made in 5 independent experiments and the SR-BI data are representative of results from 6 independent experiments using PBMCs from different donors.

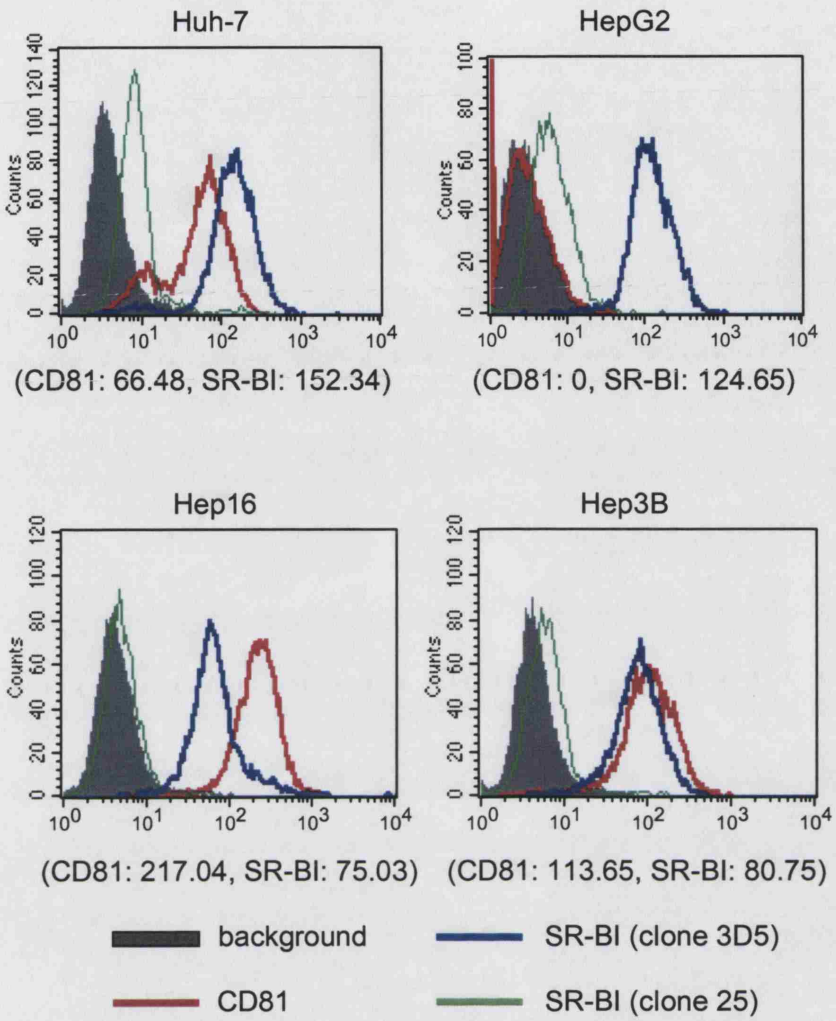


Figure 3.6. Expression of CD81 and SR-B1 on hepatocyte cell lines.

Expression of CD81 and SR-B1 on different hepatocyte cell lines (Huh-7, HepG2, Hep16, and Hep3B cells) was analysed by flow cytometry. In each panel, the grey shaded histogram represents the background staining of the cells with an isotype-matched (IgG1) control mAb followed by a FITC-conjugated secondary anti-mouse IgG Fab fragment; the red line represents staining with an anti-CD81 (clone JS-81) mAb plus the secondary mAb; the blue line represents staining with an anti-SR-B1 (clone 3D5) mAb plus the secondary mAb; and the green line represents staining with another anti-SR-BI mAb (clone 25) plus the secondary mAb. The mean fluorescence intensity (MFI) of CD81-specific and SR-BI-specific (clone 3D5) staining is shown below each histogram. The results shown are representative of findings made in 2 independent experiments.

was lower than that on Hep16 and Hep3B cells. Although all four hepatocyte cell lines were found to express SR-BI, the level of SR-BI expression was variable: Huh-7 cells expressed the highest level of SR-BI, while Hep16 cells expressed the lowest level of this molecule. SR-BI expression was readily detected using anti-SR-BI mAb 3D5, whereas much lower levels of SR-BI were detected using clone 25. This could be due to differences in the affinity of binding of these two mAbs to SR-BI, or to differences in the relative accessibility of the SR-BI epitopes they recognise on the hepatocyte surface.

The results presented here do not permit accurate comparison of the level of CD81 and SR-BI expression on PBMC subsets and hepatocyte cell lines; but it is notable that whilst CD81 expression was readily detected on both haematopoietic cells and several hepatocyte cell lines, SR-BI was much more readily detected on the hepatocyte cell lines than on monocytes and DCs. As considered in the discussion, this may have implications for the relative ease with which HCV is able to infect hepatocytes and haematopoietic cells.

Further experiments went on to address the binding of HCV E2 proteins to different PBMC subsets.

3.2.2. Transfection of cells with $E2_{660}$ plasmids to generate soluble truncated E2 proteins

Initial studies focused on the HCV genotype 1a H77c E2 glycoprotein, because a cDNA clone of this HCV strain was shown to be infectious in a chimpanzee model (Yanagi *et al.*, 1997), and many prior studies had been carried out using this clone. In parallel, an E2 glycoprotein derived from another genotype 1a HCV strain, Glasgow (Gla), was also

used. The Gla strain was cloned directly from a HCV-infected patient and this clone was found to be non-infectious (M. McElwee & R.M. Elliott; unpublished). Although H77c and Gla $E2_{660}$ proteins share 88.8 % aa homology, they have different antigenic characteristics (Fig. 3.7). Firstly, although both $E2_{660}$ proteins can be recognised by mAbs that detect linear epitopes of E2, Gla $E2_{660}$ is not recognised by mAbs directed against conformation-dependent epitopes. Further, only H77c $E2_{660}$, but not Gla $E2_{660}$, is able to bind to CD81 (Patel *et al.*, 2000). In addition, a chimeric E2 glycoprotein, C3, which is composed of aa 364-524 from Gla and aa 525-660 from H77c, was used. The advantage of C3 $E2_{660}$ was that unlike Gla $E2_{660}$, it is recognised by a wider range of anti-E2 mAbs including conformational dependent mAbs, but it is not able to bind CD81; however, it may potentially be able to bind to other host cell receptors. Gla and C3 $E2_{660}$ were used as controls to determine CD81-dependent binding of H77c $E2_{660}$ to PBMCs.

Further binding studies described later in the chapter utilised soluble truncated $E2_{661}$ proteins from two genotype 1b strains, BK and 1B12, and another genotype 1a strain, 1A14, to investigate whether there are differences between E2s from genotype 1a and 1b subtypes and/or between HCV strains of one subtype in binding to PBMCs.

293T cells were transfected with plasmids carrying $E2_{660}$ sequences from H77c, C3 and Gla (Fig. 3.7) to produce soluble E2 proteins. A plasmid carrying GFP was also transfected into 293T cells in order to examine the transfection efficiency. The proportion of the cells that absorbed plasmids was determined by detecting cells expressing GFP by flow cytometry. More than 99 % of 293T cells were successfully transfected using the GeneJuice transfection reagent (data not shown).

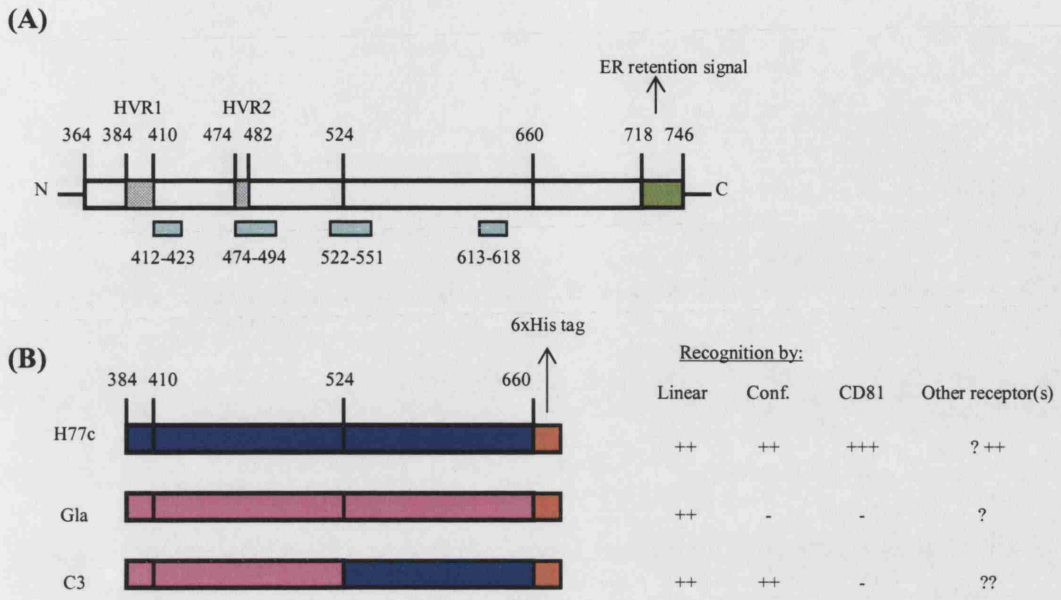


Figure 3.7. Constructs used in initial E2 binding studies in this project.

(A) shows a diagram of the full length E2 protein (aa 364-746 in the HCV polyprotein). Amino acid (aa) numbers are based on the H77c sequence. aa 364-383 correspond to the secretory signal sequence, and hypervariable regions (HVR)-1 and -2 are at aa 384-410 and aa 474-482 respectively. aa 718-746 represent the endoplasmic reticulum (ER) retention signal, removal of which results in secretion of the E2 ectodomain. Possible CD81 binding sites are indicated by blue boxes: aa 412-423 near HVR-1; HVR-2 and CD81 binding region 1 (aa 474-494, Yagnik *et al.*, (2000)); CD81 binding region 2 (aa 522-551, Yagnik *et al.*, (2000)); and aa 613-618. Three soluble E2 proteins (truncated at aa 660 ($E2_{660}$)) are shown in (B). Two of these E2 constructs were derived from infectious clone H77c and the Glasgow (Gla) strain of HCV. The chimera 3 (C3) construct carries sequences from Glasgow (aa 384-524) and H77c (aa 524-660). All constructs have a C-terminal 6 x His tag to allow protein purification. The ability of anti-E2 mAbs directed against linear and conformational (conf.) epitopes of E2 to recognise each of the truncated proteins is indicated, as is their ability to bind CD81, and their predicted ability to bind to other possible cellular receptors. (Figure adapted from Patel *et al.*, (2000))

Supernatant and cell-associated $E2_{660}$ fractions were collected after 3 days as described in the Materials and Methods. Approximately 200 ml of supernatant was concentrated down to 1-5 ml using a Centricon or a Centriprep filtration device. In order to confirm the expression of $E2_{660}$ in transfected 293T cells, $E2_{660}$ expressed in both supernatant and cell-associated fractions was analysed by SDS-PAGE and Western blotting using anti-E2 mAbs before purification (Fig. 3.8).

When expressed in *in vitro* systems, E2 tends to form disulfide-linked aggregates, which are thought to result from a non-productive folding pathway (Deleersnyder *et al.*, 1997; Dubuisson *et al.*, 1994). Non-reducing SDS-PAGE showed both monomeric and aggregated forms of $E2_{660}$ in supernatant-derived H77c, Gla and C3 preparations. Supernatant monomeric $E2_{660}$ had the expected size, ~60 kDa, in all preparations. Western blotting could not be used to provide a quantitative analysis of the ratio of aggregated to monomeric $E2_{660}$ in a particular preparation, as the amount of aggregated material was high, and complete transfer of all aggregates onto the nitrocellulose membrane usually did not occur [as residual proteins were observed in the gel by Coomassie brilliant staining (data not shown)]. However these blots showed that although a significant amount of the supernatant $E2_{660}$ from H77c and C3 migrated as a monomeric form, in the Gla preparation, only a little was monomeric. H77c and C3 preparations typically contained similar amounts of monomeric E2 protein, whereas Gla preparations contained much lower amounts of monomeric E2. The difference in the amount of monomeric E2 detected in the H77c, C3 and Gla preparations by Western blotting was not due to differential recognition of the E2 proteins by the detection mAbs. All three E2 proteins were recognised equally well by anti-E2 mAbs 3/11 and 6/53 and an anti-Penta-His mAb in Western blotting (data not shown). In addition, this result was

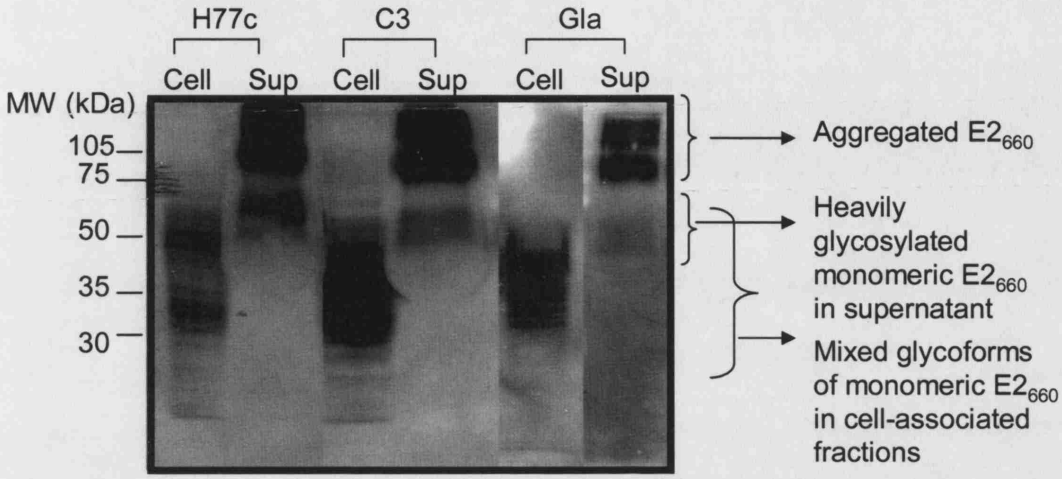


Figure 3.8. Western blot analysis of E2 expression in supernatant and cell-associated fractions from cells transfected with plasmids expressing H77c, C3 or Gla $E2_{660}$ genes.

Supernatant (Sup) and cell-associated (Cell) fractions were harvested from 293T cells transfected with plasmids encoding H77c C3 and Glasgow (Gla) $E2_{660}$ proteins. Equal amounts of total protein from these fractions were run on SDS-PAGE gels together with molecular weight (MW) markers, transferred and analysed by Western blotting. H77c, C3 and Gla $E2_{660}$ were detected using anti-E2 mAb 3/11. Bands representing aggregated $E2_{660}$ and differently glycosylated monomeric forms of $E2_{660}$ are indicated.

consistent with published data showing that E2 from the Glasgow strain is more prone to form aggregates than that of the H77c strain (Patel *et al.*, 2000).

Cell-associated H77c, C3 and Gla $E2_{660}$ proteins appeared as a large smear which had a MW of between 30-70 kDa. The smear likely represented forms of $E2_{660}$ which had acquired different amounts of glycosylation when moving through the host cell secretory pathway. Although the majority of the cell-associated $E2_{660}$ was of the expected MW, the smallest glycoforms were somewhat smaller than seen in previously published studies (Flint *et al.*, 2000). In addition, the preparations of cell-associated $E2_{660}$ did not contain aggregated forms, which were previously observed by Flint *et al.* (Flint *et al.*, 2000). It is thus possible that a proportion of the cell-associated $E2_{660}$ may have been degraded by proteases during its preparation.

These results confirmed that the supernatant and cell-associated $E2_{660}$ fractions produced from 293T cells transfected with plasmids encoding truncated E2 glycoproteins contained E2 proteins of approximately the expected sizes, which could be recognised by mAbs specific to E2.

3.2.3. Purification of $E2_{660}$ by Ni^{++} column chromatography

Each E2 protein carried 6 x His at the C-terminus to allow purification on a Ni^{++} column. Columns covered with Ni^{++} ions allow selective retention of proteins with exposed complex-forming aa residues, especially histidine. E2 proteins were purified from the six different $E2_{660}$ preparations (the supernatant and cell-associated fractions from cells transfected with the H77c, C3 or Gla $E2_{660}$ plasmids). Protein samples were applied to the column, which was then washed with buffer. Flow-through and wash fractions were collected. Retained proteins were then eluted using 1M imidazole, and the eluate was

collected in 1 ml fractions. The E2 content of all fractions was then determined by Western blotting using anti-E2 mAbs.

Fig. 3.9 shows Western blot analysis of the fractions collected during purification of H77c $E2_{660}$ from cell supernatants. The majority of the $E2_{660}$ was found in fraction one from the column eluate. Coomassie staining of a gel confirmed that most non-specific cellular proteins came out of the column in the flow-through and wash fractions (data not shown). Similar results were obtained in the purification of supernatant Gla and C3 E2s.

After purification, the total amount of protein obtained was quantified by biochemical assay. This showed that fraction one from the supernatant H77c, C3 and Gla $E2_{660}$ protein preparations contained approximately 1-2 mg/ml protein. The amount of monomeric E2 in the protein preparations was compared by running the purified samples on non-reducing SDS-PAGE gels followed by Western blotting (data not shown). It was important to compare the content of monomeric E2 in all batches of each protein preparation, since only monomeric forms of E2 can bind to CD81 (Flint *et al.*, 2000). Different preparations of purified H77c and C3 $E2_{660}$ were routinely found to contain similar amount of monomeric E2, whereas Gla $E2_{660}$ preparations always contained less monomeric $E2_{660}$ than H77c and C3 $E2_{660}$ preparations.

When cell-associated $E2_{660}$ proteins were purified on Ni^{++} columns, just weak bands of around 60 kDa and 80 kDa were detected in the column eluate by Western blotting using either anti-E2 mAbs or an anti-Penta-His mAb (data not shown). The majority of the E2 protein may thus not have bound to the column, or may have stayed on the columns. Together with the results in Fig. 3.8, this finding suggests that E2 may not

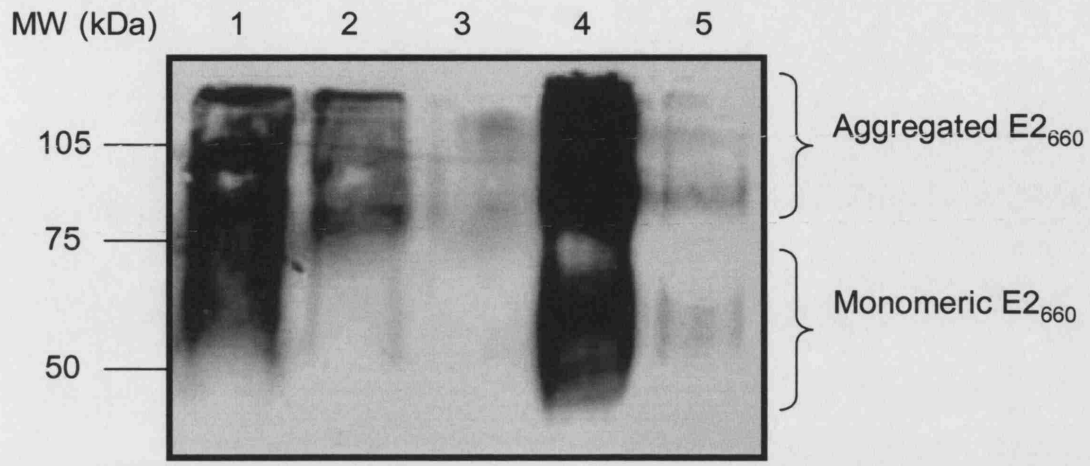


Figure 3.9. Western blot analysis of fractions collected during Ni^{++} column purification of $E2_{660}$ from cell supernatants.

Supernatant H77c $E2_{660}$ was purified using a Ni^{++} column. Fractions from all steps in the purification were collected, run on an SDS-PAGE gel together with molecular weight markers (MW), transferred and then E2-containing fractions were identified by Western blotting using anti-E2 mAb 3/11. The lanes are as follows: (1) 5 μl of starting material (concentrated cell supernatant prior to purification, a total of ~5 ml of which was applied to the column), (2) 5 μl of flow-through, from a total of ~5 ml, (3) 5 μl of wash fraction, from a total of 10 ml, (4) 5 μl of column eluate fraction one from a total of 1 ml and (5) 5 μl of column eluate fraction two from a total of 1 ml. Column eluate fractions one and two are the first two 1 ml fractions collected with elution buffer. Bands representing aggregated and monomeric E2 are indicated.

have been properly extracted from the transfected cells by the method used in this study, and/or that the cell-associated $E2_{660}$ may have been degraded during its preparation.

These results indicated that high concentrations of E2 could be purified using Ni^{++} columns from the supernatant fractions of cells transfected with H77c, C3 or Gla $E2_{660}$ plasmids. Since very little cell-associated $E2_{600}$ was obtained following Ni^{++} column purification, only purified supernatant $E2_{660}$ proteins were used in further experiments.

3.2.4. Characterisation of $E2_{660}$ by HPLC

HPLC was performed using a TSK column (on which proteins are separated according to MW) on the purified supernatant H77c and C3 E2 preparations, both in order to investigate whether they were reasonably pure, and to test the feasibility of separating monomeric and aggregated forms of E2 using this approach. After running supernatant H77c $E2_{660}$ through a TSK column, peak protein elution was observed at a time consistent with elution of proteins in the MW range between 158 and 44 kDa, which was the expected size for monomeric and aggregated $E2_{660}$ (Fig. 3.10-A). Detection of one peak suggested that there was no major cellular contamination in the preparation and a high level of purification of E2 had been achieved on the Ni^{++} column. A similar profile was obtained from HPLC analysis of supernatant C3 $E2_{660}$ (data not shown).

In order to confirm that the peak consisted of $E2_{660}$, and to address whether the aggregated and monomeric forms of E2 had been separated, fractions from the supernatant H77c $E2_{660}$ peak were analysed by Western blotting using anti-E2 mAb 3/11 (Fig. 3.10-B). The results obtained showed the aggregated form of $E2_{660}$ eluted from the column slightly faster than the monomeric form (consistent with it being of a

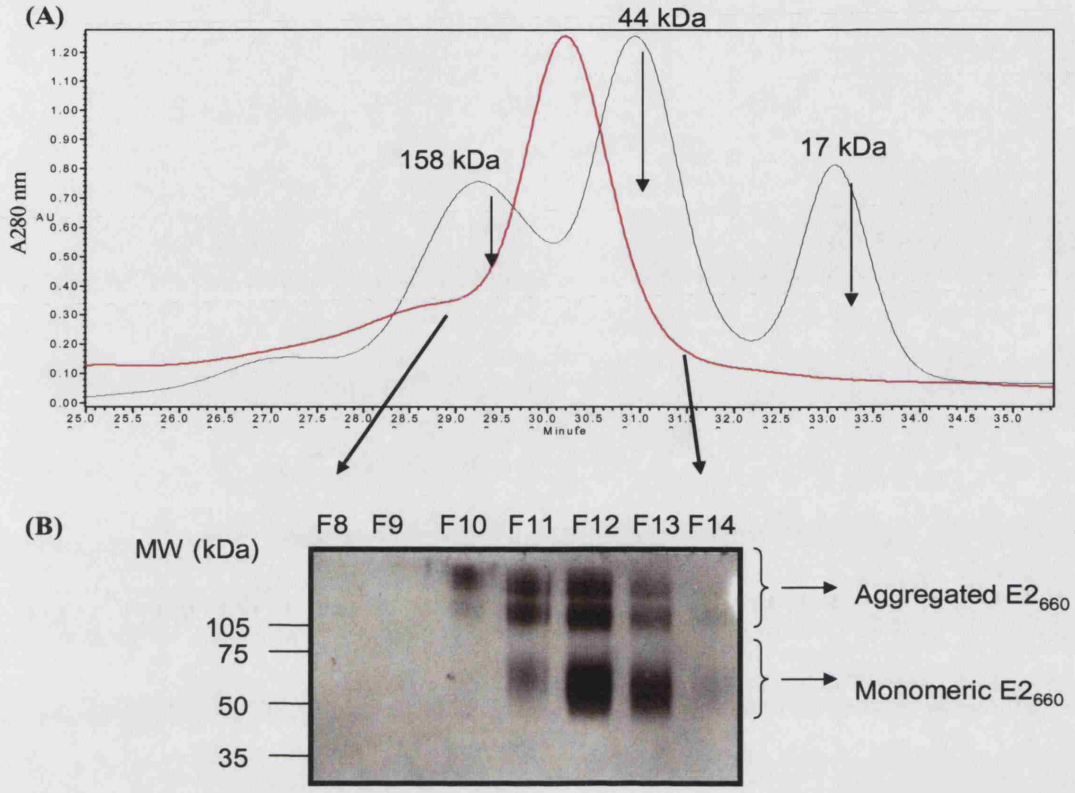


Figure 3.10. Analysis of $E2_{660}$ by HPLC on a TSK column.

Purified supernatant H77c $E2_{660}$ was separated by HPLC on a TSK column (A). Protein elution over time (minutes) was monitored spectrophotometrically; A280 values are shown. The black line represents a standard (peaks corresponding to proteins of different molecular weights (MW) are indicated) and the red line corresponds to the E2 sample. Fractions from the E2 peak (F8-F14) were run on a SDS-PAGE gel together with MW markers and Western blotted with mAb 3/11 (B). Bands corresponding to the aggregated and monomeric forms of $E2_{660}$ are indicated.

higher MW), although there was considerable overlap between the elution profiles of the two forms, indicating that the aggregated and monomeric forms of $E2_{660}$ could not readily be separated on this type of column.

3.2.5. Antigenic phenotypes of supernatant H77c, C3 and Gla $E2_{660}$ proteins

It has been reported that H77c, C3 and Gla $E2_{660}$ differ in their recognition by mAbs directed against linear and conformational E2 epitopes (Patel *et al.*, 2000). Recognition of the purified supernatant H77c, C3 and Gla $E2_{660}$ proteins by a panel of anti-E2 mAbs was tested using a GNA lectin capture EIA (Fig. 3.11). The mAbs used were described by Flint *et al.*, Clayton *et al.*, Patel *et al.* and Triyatni *et al.* (Clayton *et al.*, 2002; Flint *et al.*, 2000; Flint *et al.*, 1999b; Patel *et al.*, 2000; Triyatni *et al.*, 2002a). H77c $E2_{660}$ was recognised by all mAbs tested, including the conformation-dependent mAbs 9/27, 9/86a, H53, H50 and H33. Recognition by these mAbs is important, since they are specific for a conformation-dependent epitope on non-disulfide-bridged E2 that is expressed in complexes of E2 with E1, which are believed to represent the native form of the HCV envelope (Deleersnyder *et al.*, 1997; Flint *et al.*, 2000; Patel *et al.*, 2000). Gla $E2_{660}$ was antigenically very different from H77c $E2_{660}$. It was only recognised by mAbs 3/11, AP33 and 6/53, which recognise linear epitopes, but not by any of the conformation-dependent mAbs. C3 $E2_{660}$ was recognised by several anti-E2 mAbs, including the conformation-dependent mAbs H53, H50 and H33, which indicated that C3 $E2_{660}$ was folded correctly. However, mAbs 9/27 and 9/86a, which recognise a conformational epitope within HVR-1 (Clayton *et al.*, 2002), detected only H77c but not C3 $E2_{660}$.

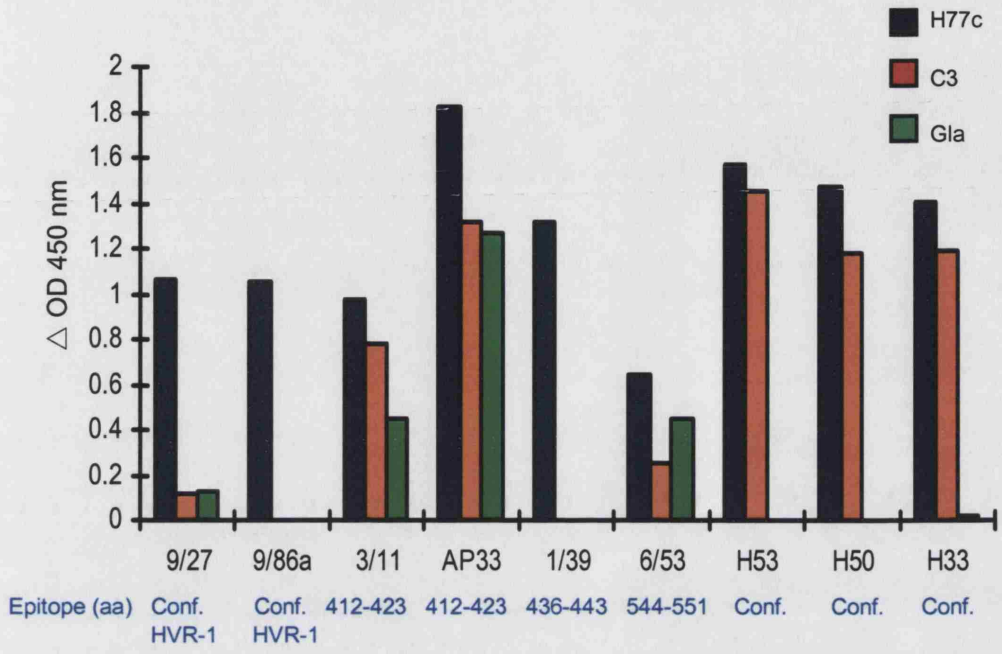


Figure 3.11. Recognition of supernatant forms of $E2_{660}$ derived from H77c, C3 and Glasgow clones by a panel of anti-E2 mAbs in a GNA lectin capture EIA.

Purified supernatant $E2_{660}$ derived from H77c (blue bars), C3 (orange bars) and Glasgow (Gla; green bars) was immobilised in GNA lectin-coated wells. E2 proteins were detected using a panel of anti-E2 mAbs (3/11, 9/27, 9/86a, 1/39, 6/53, AP33, H53, H50, and H33) and appropriate HRP-conjugated secondary mAbs, followed by a colour reaction monitored at 450 nm. The results are expressed as Δ optical density (Δ OD) values, i.e. OD value from wells to which $E2_{660}$ was added – OD value from wells to which only the detection mAbs (anti-E2 mAb and a secondary mAb) were added. Details of the epitopes recognised by each of the anti-E2 mAbs are indicated below the graph. MAbs 9/27 and 9/86a recognise epitopes within hypervariable region-1 (HVR-1). MAbs recognising conformation dependent epitopes are denoted as Conf. Numbers represent the amino acid (aa) residues within E2 that contain the epitopes recognized by mAbs recognizing linear epitopes.

3.2.6. Investigation of $E2_{660}$ binding to GNA lectin, hCD81 and mCD81

The binding of H77c $E2_{660}$ to GNA lectin (which binds to any N-linked glycoproteins) and to the CD81 LEL was then determined by EIA. Dose-dependent binding of purified supernatant H77c $E2_{660}$ to both GNA lectin and to hCD81 was observed; there was also a low level of background binding to mCD81 (Fig. 3.12). Supernatant C3 $E2_{660}$ also bound to GNA lectin at the same level as H77c $E2_{660}$, although as expected, binding of C3 $E2_{660}$ to hCD81 was not observed, even at the highest E2 concentrations (data not shown).

As GNA lectin binds to both the aggregated and monomeric forms of E2, whereas only the monomeric form of E2 can bind to hCD81, the relative levels of GNA-lectin-binding to hCD81-binding protein can thus be used to give an estimate of the relative quantities of aggregated and monomeric forms of E2 in purified preparations of H77c $E2_{660}$. At 1 and 2 μg/well of H77c $E2_{660}$, binding to GNA lectin seemed to be saturated. Although the range of E2 concentrations tested were not high enough to saturate binding to hCD81, making it difficult to determine the exact ratio between aggregated and monomeric $E2_{660}$, these results show that the amount of aggregated $E2_{660}$ was considerably higher than that of monomeric $E2_{660}$ (likely around 5-10 fold higher).

In summary, the protein preparations of supernatant H77c, C3 and Gla $E2_{660}$ contained both monomeric and aggregated forms of E2. H77c and C3 $E2_{660}$ were folded properly, and the former bound to hCD81.

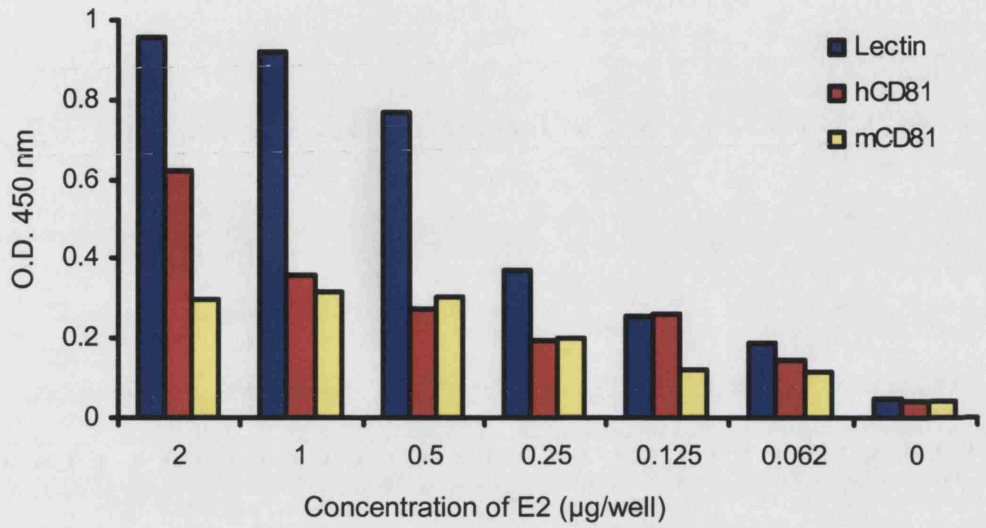


Figure 3.12. Binding of H77c $E2_{660}$ to GNA lectin, hCD81 and mCD81 as assessed by EIA.

The binding of purified supernatant H77c $E2_{660}$ to GNA lectin, human CD81 (hCD81) and mouse CD81 (mCD81) was measured by EIA. The indicated concentrations of H77c $E2_{660}$ were incubated in plates coated with GNA lectin (blue bars), hCD81 (red bars) or mCD81 (blue bars). E2 binding was detected using mAb H53 and a HRP-conjugated secondary mAb, followed by a colour reaction monitored at 450 nm. The results shown are the optical density (OD) values obtained for wells to which different concentrations of E2 were added.

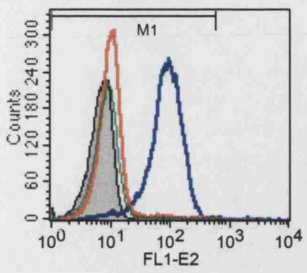
3.2.7. H77c, C3 and Gla $E2_{660}$ binding to hepatocyte cell lines

Further experiments addressed the binding of H77c, C3 and Gla E2 proteins to different cell types. Cells were incubated with $E2_{660}$, and E2 binding was detected using an anti-Penta-His mAb or a conformation dependent anti-E2 mAb, H53, which has been shown to recognise E2 bound to CD81 on the cell surface (Cocquerel *et al.*, 1998; Flint *et al.*, 1999b). Gla $E2_{660}$ was not recognised by mAb H53 (Fig. 3.11), thus the anti-His mAb was used when the binding of H77c, C3 and Gla $E2_{660}$ was compared. The level of E2 binding to gated cells was analysed as described in Fig. 3.13.

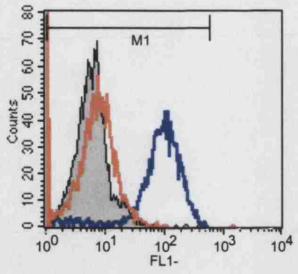
As the liver is the primary infectious site for HCV, the interaction between E2 proteins and hepatocyte cell lines has been investigated in a number of previous studies. To confirm whether the purified supernatant H77c, C3 and Gla $E2_{660}$ I had produced could bind to hepatocytes, E2 binding to HepG2 and Hep16 cells was analysed. H77c and C3 $E2_{660}$ both bound to HepG2 cells, which express SR-BI but not CD81, suggesting that although C3 does not bind to CD81, it is able to bind to SR-BI and/or other receptor(s) on the hepatocyte cell surface (Fig. 3.14-A). H77c and C3 $E2_{660}$ also bound to Hep16 cells, which expressed both CD81 and SR-BI, suggesting that the levels of SR-BI and/or other receptors were sufficiently high to saturate E2 binding (Fig. 3.14-B). The binding of Gla $E2_{660}$ to Hep16 cells was scarcely detectable, which is likely due to there being much lower amounts of monomeric E2 in the Gla $E2_{660}$ preparations than the H77c and C3 $E2_{660}$ preparations (Fig. 3.14-B).

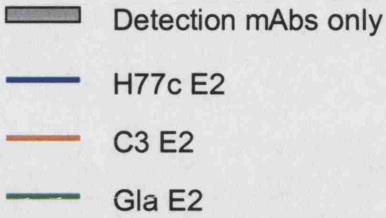
These results indicated that soluble truncated H77c and C3 E2 proteins can bind to hepatocyte cell lines, and that both the anti-E2 mAb H53 and an anti-Penta-His mAb were able to recognise not only E2 complexed to CD81, but also to SR-BI/other receptor(s). In addition, they showed that although C3 $E2_{660}$ cannot bind to CD81

(A)



(B)





(C)

Detection mAbs only

Marker	% Gated	Mean
All	100.00	6.62
M1	98.86	6.69

H77c E2

Marker	% Gated	Mean
All	100.00	97.06
M1	99.16	97.87

C3 E2

Marker	% Gated	Mean
All	100.00	8.46
M1	98.64	8.36

(D)

MFI of E2-specific binding = The mean of E2 binding – The mean of detection mAbs

H77c E2-specific binding = 97.87 – 6.69 = 91.18
C3 E2-specific binding = 8.36 – 6.69 = 1.67

Figure 3.13. Analysis of E2 binding to cells.

E2 proteins were incubated with PBMCs, monocyte-derived DCs or other cell lines, and bound E2 was detected using either anti-E2 mAb H53 followed by a FITC-conjugated secondary mAb or an Alexa fluor 488-conjugated anti-Penta-His mAb. PBMC subsets were identified by co-staining with mAbs against surface markers as described in the legend to Figure 3.1 and E2 binding to the gated cells was analysed. The histograms in (A) and (B) show E2 binding to NK cells detected using the anti-His mAb (A) or anti-E2 mAb H53 (B). The grey shaded histogram represents the background binding of the detection mAb(s) to cells in the absence of E2, and the other lines represent staining of NK cells after incubation of PBMCs with H77c E2 (blue line), C3 E2 (orange line), or Gla E2 (green line). (C) shows how the mean fluorescence intensity (MFI) of E2-specific binding was calculated. The marker M1 was set to cover all cells. The MFI of E2-specific staining of cells within the marker was calculated as the MFI of cells incubated with E2 and then mAb H53 plus a secondary mAb minus the MFI of cells incubated with the detection mAbs alone. Examples of calculation of the MFI of H77c and C3-specific staining are shown in (D).

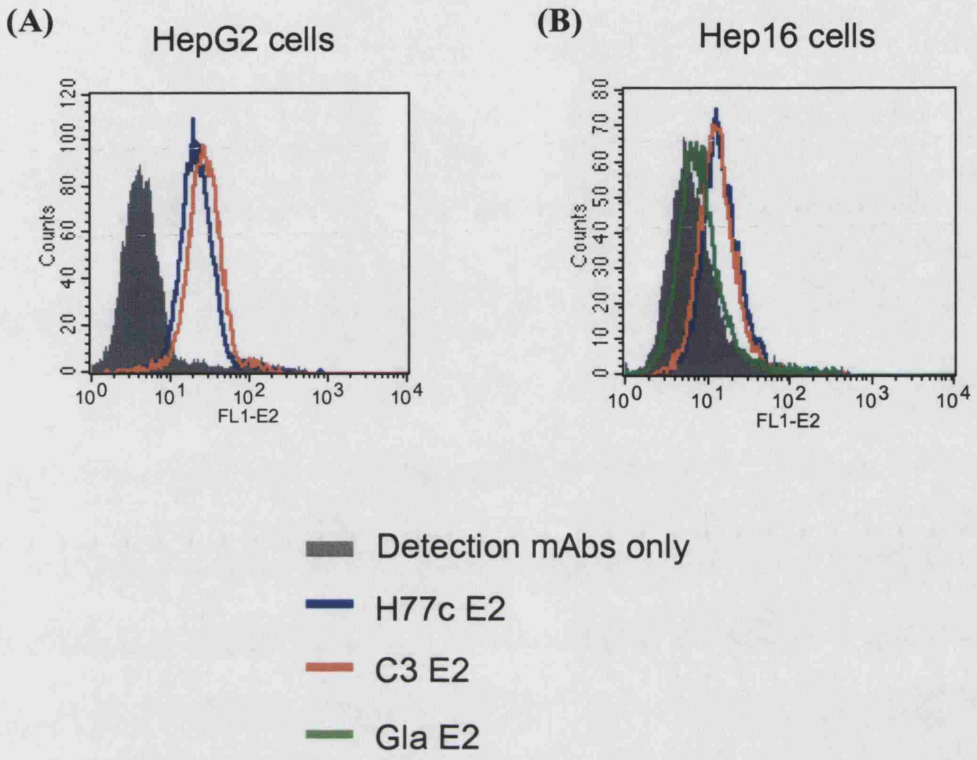


Figure 3.14. Binding of H77c and C3 $E2_{660}$ to hepatocyte cell lines.

70 μg of supernatant H77c, C3, or Glasgow (Gla) $E2_{660}$ was incubated with hepatocyte cell lines (HepG2 (A) or Hep16 (B)), and bound E2 was detected using anti-E2 mAb H53 followed by a FITC-conjugated secondary mAb (A) or an Alexa fluor 488-conjugated anti-Penta-His mAb (B). In each panel, the grey shaded histogram represents the background staining of cells with the detection mAbs in the absence of E2, and the line graphs represent the staining of cells incubated with either H77c (blue lines), C3 (orange lines), or Gla (green lines) $E2_{660}$. The data shown are representative of findings made in 3 independent experiments.

(Patel *et al.*, 2000), it can bind to SR-BI or other receptor(s) expressed on the surface of hepatocyte cell lines.

3.2.8. H77c $E2_{660}$ binding to different PBMC subsets

H77c E2 binding to lymphocyte cell lines such as Daudi (a B cell line) and Molt-4 (a T cell line) has previously been investigated (Flint *et al.*, 1999b; Roccasecca *et al.*, 2003; Wellnitz *et al.*, 2002); however, no previous studies have fully characterised the binding of E2 proteins to different PBMC subsets. To investigate whether E2 binds to PBMCs in a specific, saturable manner, PBMCs were initially incubated with various concentrations of H77c $E2_{660}$, and E2 binding was detected using conformation dependent anti-E2 mAb H53. The binding of H77c $E2_{660}$ to total PBMCs was found to be dose-dependent and saturable (Fig. 3.15-A). Likewise, when binding to NK, NT and T cells was analysed, this was also found to be dose-dependent and saturable (Fig. 3.15-B). NT cells express higher levels of CD81 on their surface than NK and T cells (Fig. 3.3-B). In parallel, the level of H77c $E2_{660}$ binding to NT cells was higher than that observed to NK or T cells.

E2 binding to multiple different PBMC subsets was investigated (Fig. 3.16). PBMCs were incubated with a sub-saturating concentration of H77c or C3 $E2_{660}$, and E2 binding was detected using mAb H53. C3 $E2_{660}$ was included in the experiment to allow assessment of CD81-independent binding of E2 to PBMCs. PBMCs were also incubated with the detection mAbs only to determine the background level of Ab binding to PBMC subsets. H77c $E2_{660}$ bound to all PBMC subsets investigated, although the level of binding to different cell types was very variable. H77c $E2_{660}$ bound to NT cells and activated $CD8^+$ T cells at a high level, whereas its binding to plasmacytoid DCs and granulocytes was very low. Interestingly, the pattern of H77c

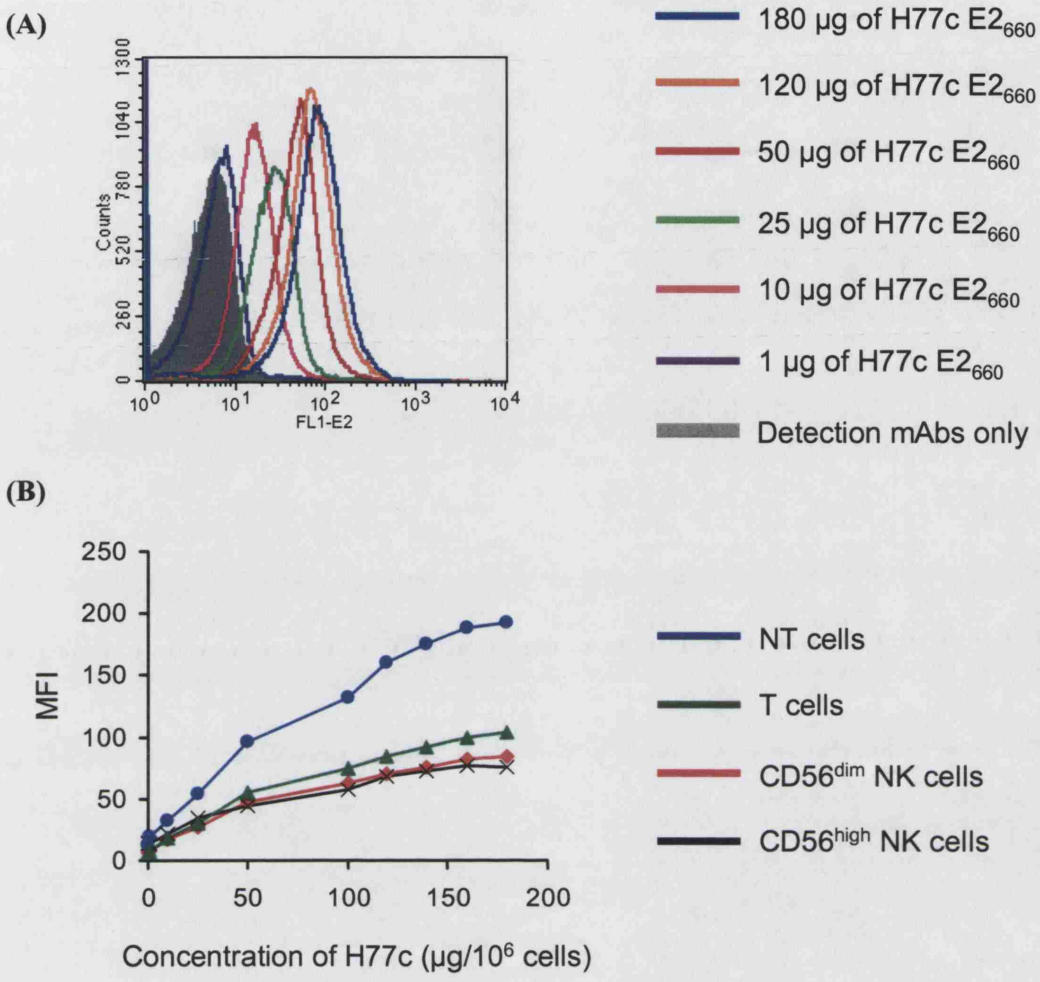


Figure 3.15. Dose-dependent, saturable binding of H77c $E2_{660}$ to PBMC subsets.

Supernatant H77c $E2_{660}$ was incubated with PBMCs and bound E2 was detected using anti-E2 mAb H53 followed by a FITC-conjugated secondary mAb. NK cell subsets, NT and T cells were identified by co-staining with mAbs against CD3 and CD56. (A) Dose-dependent binding of H77c $E2_{660}$ to total PBMCs. The grey shaded histogram represents the background binding of the detection mAbs to PBMCs in the absence of E2, and the line graphs represent staining of PBMCs incubated with different concentrations of H77c $E2_{660}$: from left to right 1 µg, 10 µg, 25 µg, 50 µg, 120 µg and 180 µg. (B) Dose-dependent, saturable binding of H77c $E2_{660}$ to NT, T, $CD56^{dim}$ NK and $CD56^{high}$ NK cells. The results shown are the mean fluorescence intensity (MFI) of staining of cells of each subset after incubation of PBMCs with the indicated concentrations of E2, and are representative of findings made in 3 independent experiments.

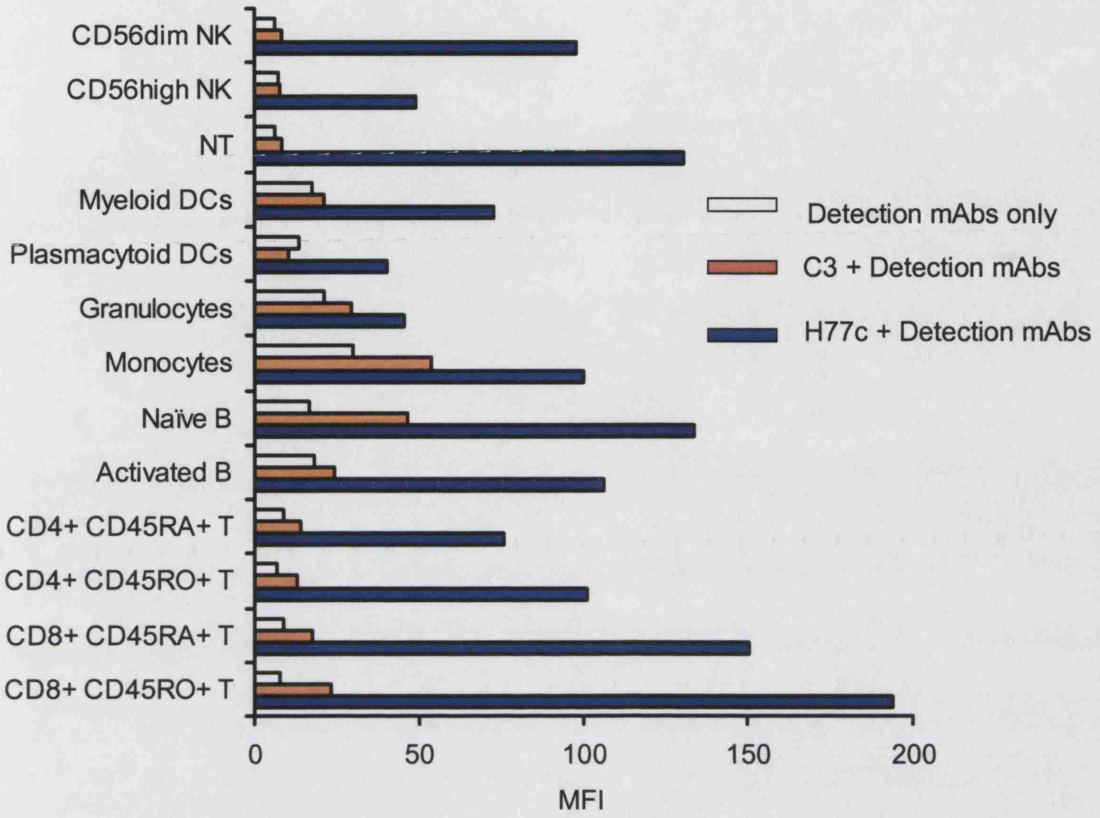


Figure 3.16. Binding of H77c and C3 $E2_{660}$ to PBMC subsets.

70 μg of purified supernatant H77c or C3 $E2_{660}$ was incubated with PBMCs and bound E2 was detected using anti-E2 mAb H53 followed by a FITC-conjugated secondary mAb. PBMC subsets were identified by co-staining with mAbs against surface markers as described in the legend to Fig. 3.1 and E2 binding was analysed as described in the legend to Fig. 3.13. The results shown are the mean fluorescence intensity (MFI) of staining of each cell subset after incubation of PBMCs with H77c (blue bars), C3 $E2_{660}$ (orange bars) or detection mAbs (mAb H53 and a secondary mAb) only. The data shown are representative of findings made in 7 independent experiments using PBMCs isolated from different donors.

binding to different PBMC subsets largely mirrored the level of CD81 they expressed, e.g. H77c E2 binding to NT cells, which expressed high levels of CD81, was high, while H77c E2 binding to plasmacytoid DCs, which expressed low levels of CD81, was low. C3 $E2_{660}$ also bound to PBMCs, although the level of binding was much lower than that of H77c $E2_{660}$. Interestingly, although C3 E2 binding to most cell subsets was scarcely above background, higher levels of C3 binding were detected to monocytes and B cells. These PBMC subsets thus exhibited the highest CD81-independent E2-binding capacity. In some experiments, the binding of Gla $E2_{660}$ to PBMCs was also analysed using an anti-Penta-His mAb (data not shown). Gla $E2_{660}$ did not show any specific binding to PBMCs; the MFI values I obtained for Gla $E2_{660}$ were almost the same as the background. This is likely because there was very little monomeric E2 in the Gla $E2_{660}$ preparations. These results were confirmed using several different batches of each of the $E2_{660}$ proteins.

3.2.9. Blocking of H77c $E2_{660}$ binding to PBMCs

The results in Fig. 3.16 suggest that the majority of H77c binding to most PBMC subsets was mediated via interaction with CD81. The dependence of H77c $E2_{660}$ binding to PBMC subsets on CD81 was further examined by testing the ability of anti-E2 mAb H33, a conformation dependent anti-E2 mAb that is known to block H77c $E2_{660}$ binding to CD81 (Patel *et al.*, 2000), or an anti-CD81 mAb to block the interaction.

Pre-incubation of H77c $E2_{660}$ with mAb H33 reduced H77c E2 binding to all PBMC subsets (Fig. 3.17). H77c binding to NK and T cell subsets was blocked almost entirely by mAb H33. However, E2 binding to monocytes, plasmacytoid DCs and B cells was not blocked as completely as that to NK/T cells. These results were consistent with much of E2 binding to PBMCs being mediated via CD81, but some cell subsets also

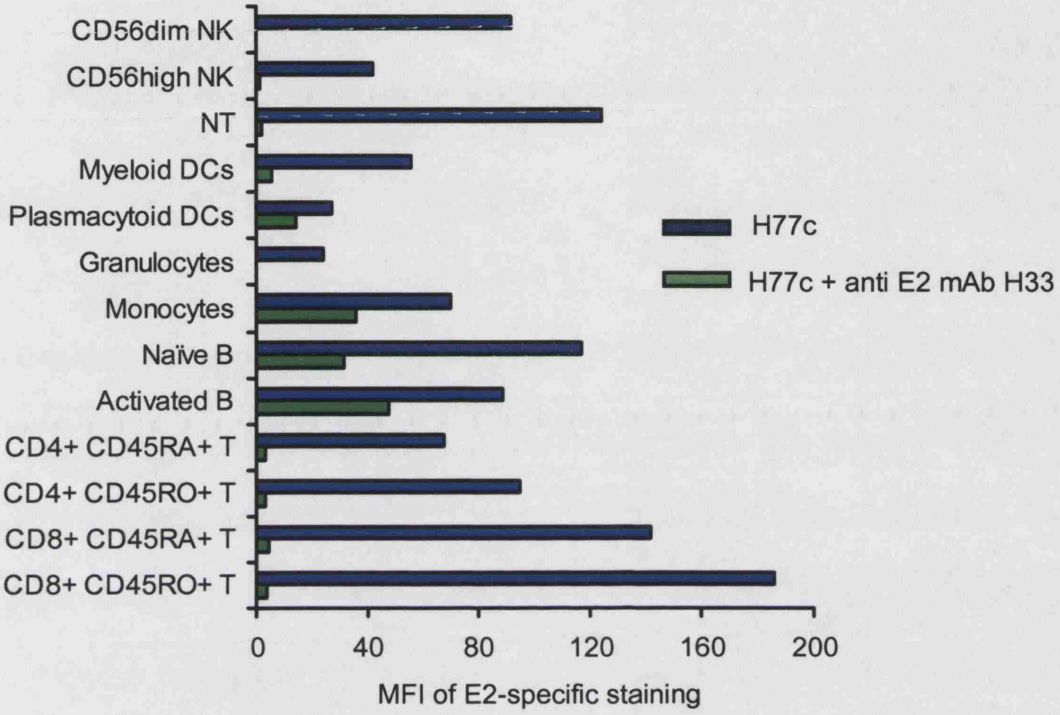


Figure 3.17. Blocking of H77c $E2_{660}$ binding to PBMC subsets by anti-E2 mAb H33.

70 μg of purified supernatant H77c $E2_{660}$ was pre-incubated for 1 hour without (blue bars) or with (green bars) 34 μg of mAb H33, then its binding to PBMC subsets was assessed by staining with anti-E2 mAb H53 followed by a secondary FITC-conjugated mAb. The results are expressed as the mean fluorescence intensity (MFI) of E2-specific staining of each PBMC subset (calculated as described in the legend to Fig. 3.13) with or without mAb pre-treatment. The data shown are representative of findings made in 3 independent experiments using PBMCs isolated from different donors.

binding E2 by means of alternative receptors.

As Fig. 3.16 shows, C3 $E2_{660}$ bound poorly to most PBMC subsets, but bound slightly better to monocytes and B cells. When C3 E2 proteins were pre-incubated with mAb H33, C3 $E2_{660}$ binding to NK/T cell subsets was blocked completely, whereas the binding of C3 $E2_{660}$ to monocytes and B cells could not be blocked fully (data not shown). Although it is unclear whether mAb H33 can interfere with the interaction between E2 and receptors other than CD81, the failure of this mAb to block C3 binding to monocytes/B cells suggests that mAb H33 is unlikely to block E2 binding to other receptors.

To address the CD81 dependence of H77c E2 binding to PBMC subsets more directly, the ability of an anti-CD81 mAb to block H77c E2 binding was analysed (Fig. 3.18). E2 binding to NK, NT and T cells was blocked well, whereas E2 binding to monocytes, plasmacytoid DCs, granulocytes and B cells was blocked less completely. Similar results were obtained in experiments using anti-CD81 mAbs JS-81 and 1.3.3.22.

These results again suggest that much of the interaction of H77c $E2_{660}$ with PBMC subsets is mediated through CD81, and that while E2 binding to some PBMC subsets, e.g. NK/T cells, is almost completely dependent on CD81, others, e.g. monocytes and B cells, have a greater CD81-independent binding component. Notably, the PBMC subsets with the highest CD81-independent binding capacity are those reported to be sites of HCV infection *in vivo*. This is discussed further at the end of the chapter.

3.2.10. Binding of H77c $E2_{660}$ to monocyte-derived DCs

It has been shown that monocyte-derived DCs can be infected by HCV from patient serum samples and are capable of supporting viral replication (Navas *et al.*, 2002). This indicates that monocyte-derived DCs express receptors that enable HCV to enter the

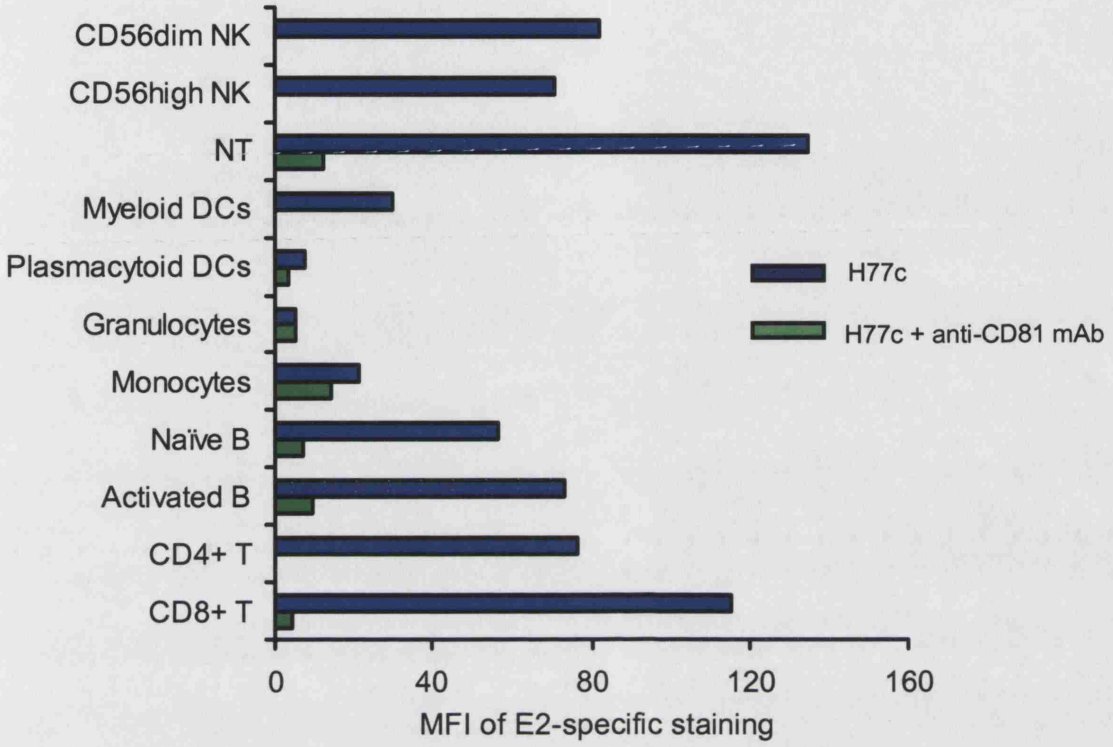


Figure 3.18. Blocking of H77c $E2_{660}$ binding to PBMC subsets by anti-CD81 mAb.

PBMCs were incubated without (blue bars) or with (green bars) anti-CD81 mAb (clone JS-81) prior to the addition of 70 μg supernatant H77c $E2_{660}$. E2 binding was assessed by staining with Alexa fluor 488-conjugated anti-E2 mAb H53. The results are expressed as mean fluorescence intensity (MFI) of E2-specific staining of each subset (calculated as described in the legend to Fig. 3.13) with or without mAb pre-treatment, and are representative of findings made in 2 independent experiments using PBMCs isolated from different donors.

cells. In line with this, I found that monocyte-derived DCs expressed both CD81 and SR-BI (Fig. 3.5). To explore the association between susceptibility of cells to infection and their ability to bind E2 by both CD81-dependent and -independent means further, the binding of H77c $E2_{660}$ to monocyte-derived DCs was investigated. H77c $E2_{660}$ bound to monocyte-derived DCs (Fig. 3.19-A). C3 $E2_{660}$ also bound to monocyte-derived DCs although the level of binding was much lower than that of H77c (data not shown). The dependence of H77c $E2_{660}$ binding on CD81 was also examined by testing the ability of anti-E2 mAb H33 or an anti-CD81 mAb to block the binding of this protein to monocyte-derived DCs (Fig. 3.19-B). Approximately 65 % of H77c binding to monocyte-derived DCs was inhibited by pre-incubation of E2 with mAb H33 or pre-incubation of monocyte-derived DCs with the anti-CD81 mAb, and the residual binding could not be eliminated with saturating amount of blocking mAbs. These results indicate that monocyte-derived DCs are another cell type that H77c E2 binds to via both CD81 and alternative receptors.

3.2.11. Investigation of the role played by SR-BI in H77c $E2_{660}$ binding to monocytes

The observation that a component of H77c $E2_{660}$ binding to certain PBMC subsets was independent of CD81 suggested the involvement of other receptors in mediating E2 binding to these cell types. Many of cell subsets exhibiting CD81-independent E2 binding expressed SR-BI; I thus investigated the contribution of SR-BI to CD81-independent E2 binding to monocytes.

PBMCs were incubated with an anti-CD81 mAb, an anti-SR-B1 mAb, an isotype control mAb, or with a combination of anti-CD81 and anti-SR-BI mAbs before E2 proteins were added, and then E2 binding to $CD14^{high}$ gated cells (monocytes) was

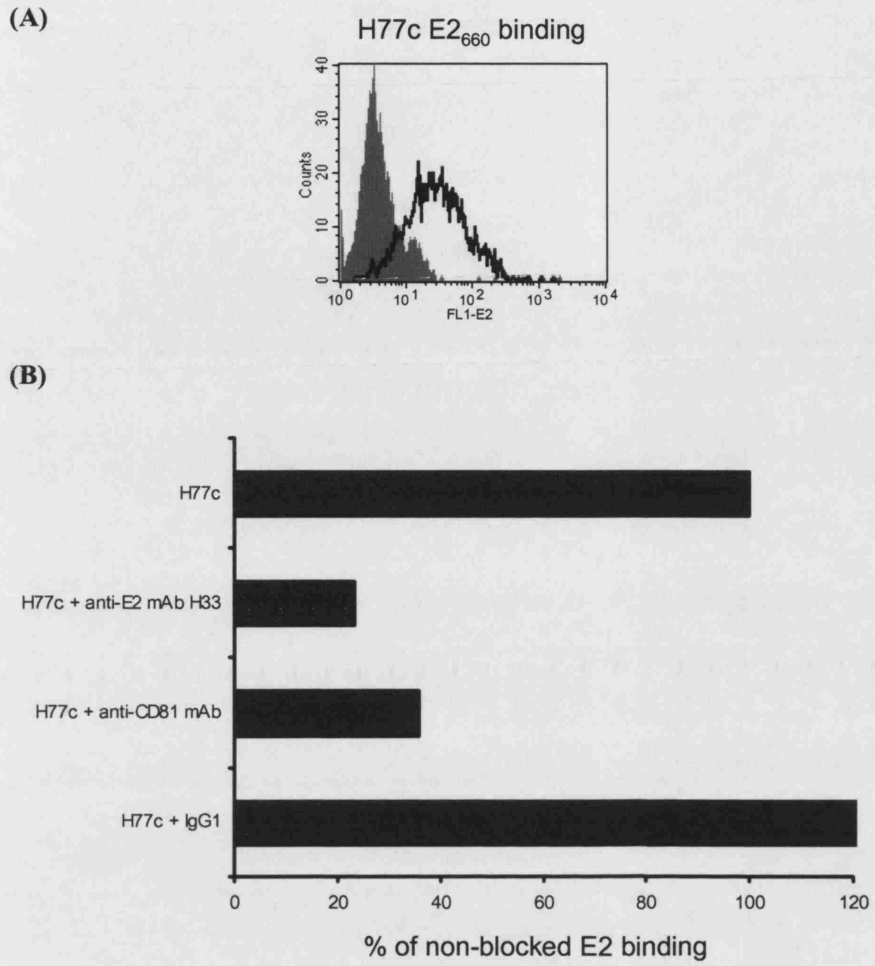


Figure 3.19. Binding of H77c $E2_{660}$ to monocyte-derived DCs.

70 μg of purified supernatant H77c $E2_{660}$ was incubated with monocyte-derived DCs and bound E2 was detected using anti-E2 mAb H53 followed by a FITC-conjugated secondary mAb (A). The grey shaded histogram represents the background binding of the detection mAbs to monocyte-derived DCs in the absence of E2, and the line graph represents staining of monocyte-derived DCs after incubation of cells with 70 μg of H77c $E2_{660}$ followed by the detection mAbs. The data shown are representative of findings made in 4 independent experiments. (B) Blocking of H77c $E2_{660}$ binding to monocyte-derived DCs by anti-E2 mAb H33 or anti-CD81 mAb. This was analysed as described in the legends to Figures 3.17 and 3.18. E2 binding to monocyte-derived DCs after treatment with anti-E2 mAb H33 or anti-CD81 mAb is expressed as a percentage of the specific binding of E2 to these cells in the absence of blocking mAbs (100 %). The data shown are representative of findings made in 3 independent experiments using PBMCs from different donors.

analysed. The anti-CD81 mAb inhibited >50 % of E2 binding to monocytes, but the binding was not completely blocked (Fig. 3.20). The anti-SR-BI mAb also reduced E2 binding to monocytes, showing that this molecule also played a role in E2 binding to these cells, although its contribution was smaller than that of CD81. When used in combination, the anti-CD81 and SR-BI mAbs blocked H77c binding to monocytes in an additive fashion. These results indicate that both CD81 and SR-BI contribute to the interaction between H77c $E2_{660}$ and monocytes. Some residual binding of E2 still occurred after incubation of the cells with anti-CD81 and anti-SR-BI blocking mAbs, which could not be eliminated with saturating amount of both mAbs. One possible explanation for this observation is that additional receptor component(s) may contribute to the interaction between H77c $E2_{660}$ and monocytes. However, it needs to be considered that the efficiency with which antibodies block access to receptors is a function of the dynamics of antigen binding (i.e. the on-rate and off-rate) – thus the residual E2 binding may reflect unblocked interaction with CD81 and SR-BI. Hence, on the basis of this data, it cannot be definitely concluded that there is an additional receptor for E2 on monocytes.

3.2.12. Comparison of the binding of H77c E2 and a genotype 1b E2 (BK) to PBMC subsets

It has been reported that there is heterogeneity in the interaction of genotype 1 E2 proteins with CD81. Notably E2s from several 1b clones have been shown to bind CD81 much less well than H77c (Roccasecca *et al.*, 2003; Scarselli *et al.*, 2002; Triyatni *et al.*, 2002a; Yagnik *et al.*, 2000). Since the interaction of H77c $E2_{660}$ with PBMCs was largely mediated through CD81, I carried out further experiments to investigate the binding of E2 proteins from other genotype 1 strains to PBMC subsets.

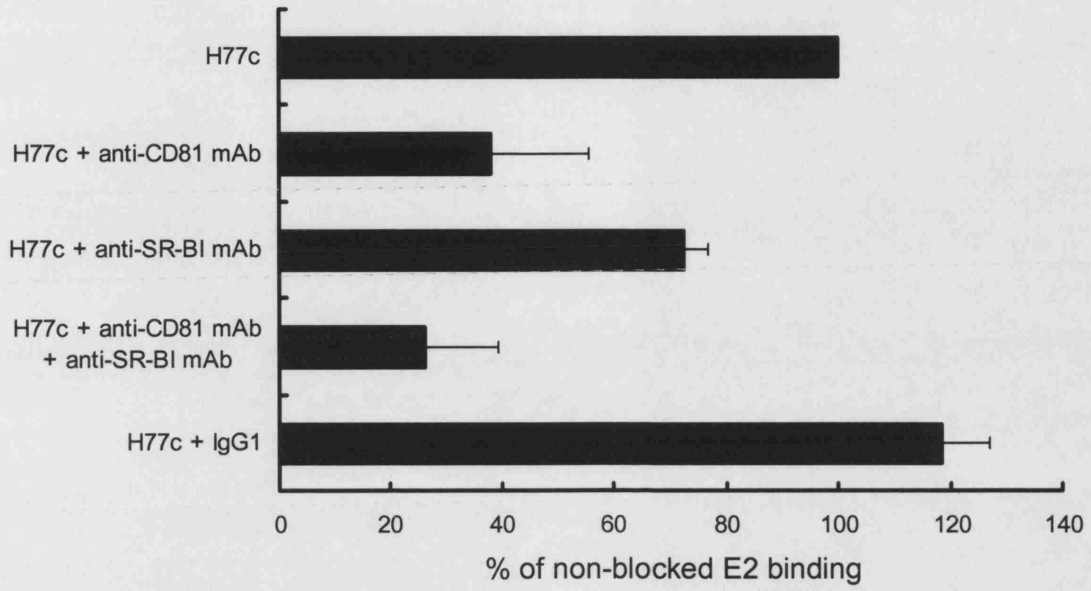


Figure 3.20. Blocking of H77c $E2_{660}$ binding to monocytes by anti-CD81 and anti-SR-BI mAbs.

PBMCs were incubated with 0.5 μg of anti-CD81 mAb (clone JS-81), 10 μg of anti-SR-BI mAb (clone 3D5), 10 μg of an isotype matched-control mAb (IgG1), or with a combination of anti-CD81 and anti-SR-BI mAbs, prior to the addition of 70 μg of H77c $E2_{660}$. E2 binding was then assessed using Alexa fluor 488-conjugated anti-E2 mAb H53, and cells were co-stained with an anti-CD14 mAb to allow identification of monocytes. The mean fluorescence intensity (MFI) of E2-specific staining of monocytes with or without antibody pre-treatment was calculated as described in the legend to Fig. 3.13, and E2 binding to antibody pre-treated cells then expressed as a percentage of E2 binding to monocytes in the absence of blocking mAbs (100 %). The data shown are the mean of results obtained in 3 independent experiments; the error bars indicate one standard error above the mean.

I initially produced soluble truncated $E2_{661}$ protein from the genotype 1b clone BK, described by Roccasecca *et al.* (Roccasecca *et al.*, 2003). Prior to investigating its binding to cells, the amount of monomeric $E2_{660/661}$ in supernatant H77c and BK preparations was compared by running samples of each purified E2 on a non-reducing SDS-PAGE gel followed by Western blotting using an anti-Penta-His mAb (Fig. 3.21). As shown in Fig. 3.21, the H77c and BK $E2_{660/661}$ protein preparations contained similar amounts of monomeric E2.

Binding of H77c and BK $E2_{660/661}$ to GNA lectin and to CD81 was also determined by EIA. Purified supernatant H77c and BK E2 proteins both bound to GNA lectin in a dose-dependent fashion (Fig. 3.22-A). However, whilst dose-dependent binding of H77c $E2_{660}$ to hCD81 was observed, BK $E2_{661}$ showed very low binding to hCD81 (Fig. 3.22-B).

As I did not have access to recombinant SR-BI with which to set up an EIA to determine the ability of E2 proteins to bind SR-BI, the binding of H77c and BK $E2_{660/661}$ to HepG2 cells, which express SR-BI but not CD81 (Fig. 3.6), and also other hepatocyte cell lines was determined. Since the conformation-dependent mAb H53 is reported not to recognise genotype 1b E2s (Bartosch *et al.*, 2003a; Bartosch *et al.*, 2003b), an anti-Penta His mAb was used to compare the binding of H77c and BK E2 proteins to hepatocyte cell lines. BK $E2_{661}$ bound to HepG2 cells at an equivalent level to H77c $E2_{660}$ (Fig. 3.23-A). As HepG2 cells do not express CD81, these results indicate that BK $E2_{661}$ binds to SR-BI and/or other receptor(s) at an equivalent level to H77c $E2_{660}$ even though BK $E2_{661}$ shows much weaker affinity to CD81 compared with H77c $E2_{660}$. At the sub-saturating protein concentrations used in this and other experiments,

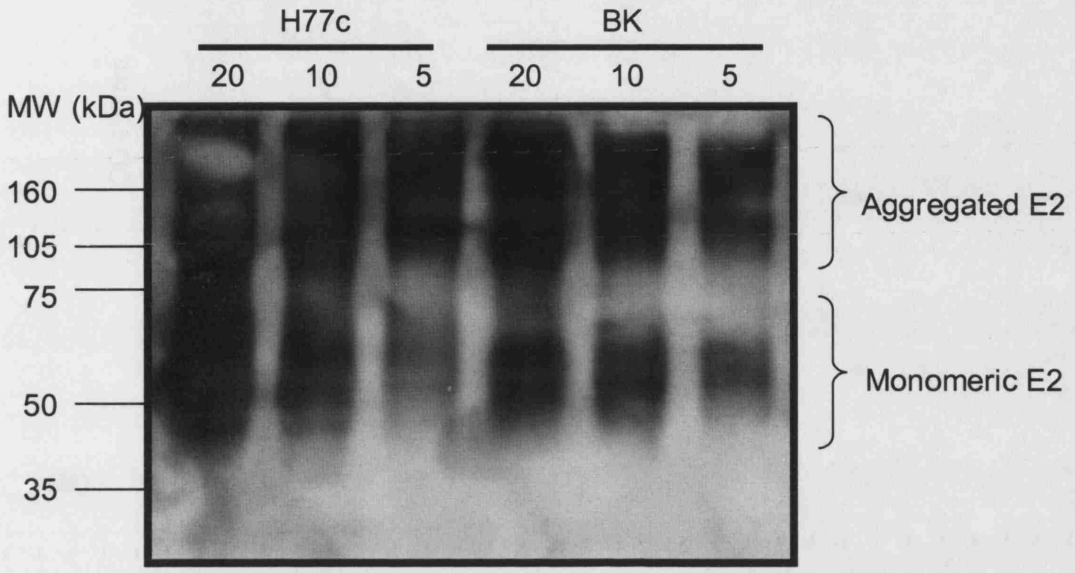


Figure 3.21. Western blot analysis of supernatant $E2_{660/661}$ from H77c and BK HCV strains.

Supernatants were harvested from 293T cells transfected with H77c or BK $E2_{660/661}$ plasmids and E2 was purified using Ni^{++} columns. Various quantities of purified $E2_{660/661}$ (from left to right 20 μg, 10 μg, or 5 μg of H77c $E2_{660}$ or BK $E2_{661}$) were run on SDS-PAGE gels together with molecular weight markers (MW), transferred and E2 was identified by Western blotting using an anti-Penta-His mAb. Bands representative of aggregated and monomeric forms of $E2_{660/661}$ are indicated.

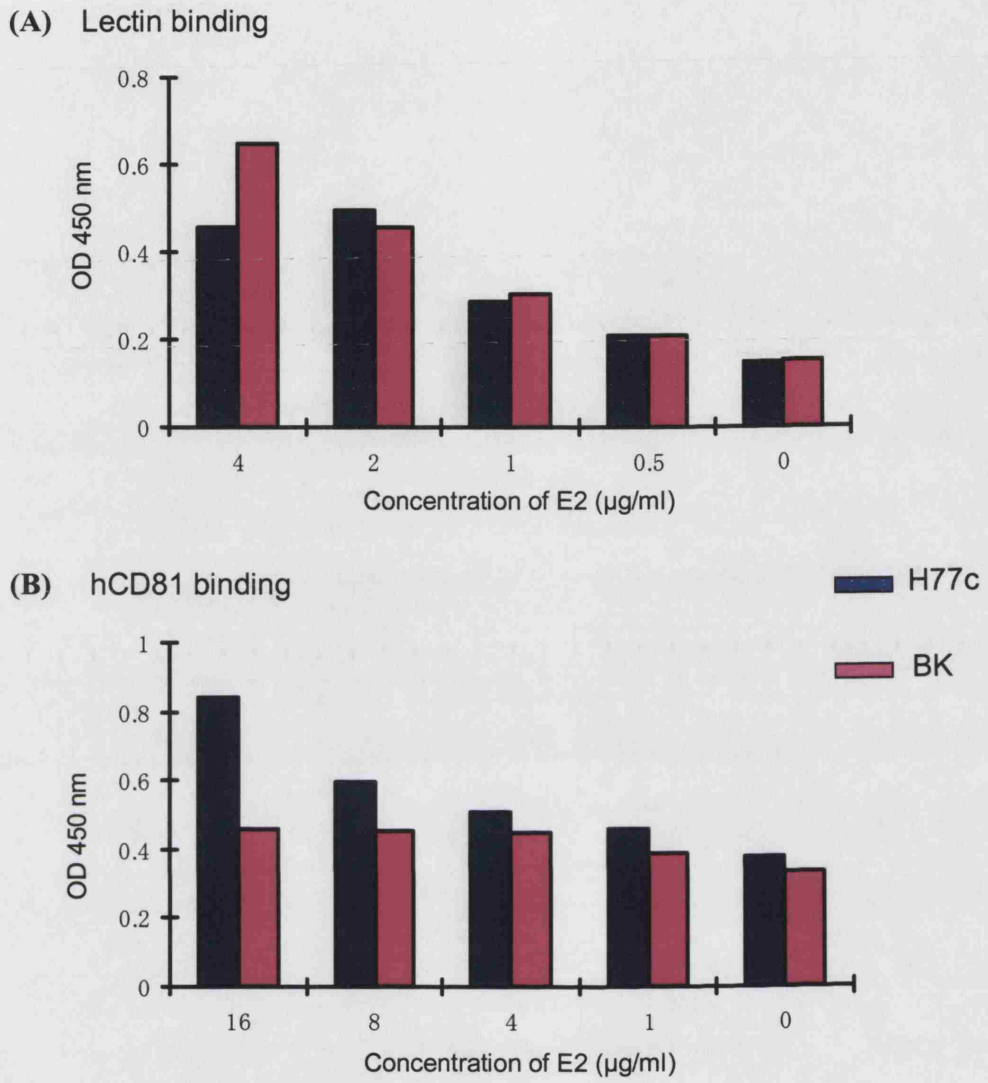


Figure 3.22. Binding of H77c and BK $E2_{660/661}$ to GNA lectin and hCD81 as assessed by EIA.

The binding of purified supernatant H77c and BK $E2_{660/661}$ to GNA lectin and human CD81 (hCD81) was measured by EIA. The indicated concentrations of H77c (blue bars) or BK $E2_{660/661}$ (pink bars) were added to plates coated with GNA lectin (A) or hCD81 (B). E2 binding was detected using an anti-Penta-His mAb and a HRP-conjugated secondary mAb followed by a colour reaction monitored at 450 nm. The results shown are the optical density (OD) values obtained for wells to which different concentrations of E2 were added.

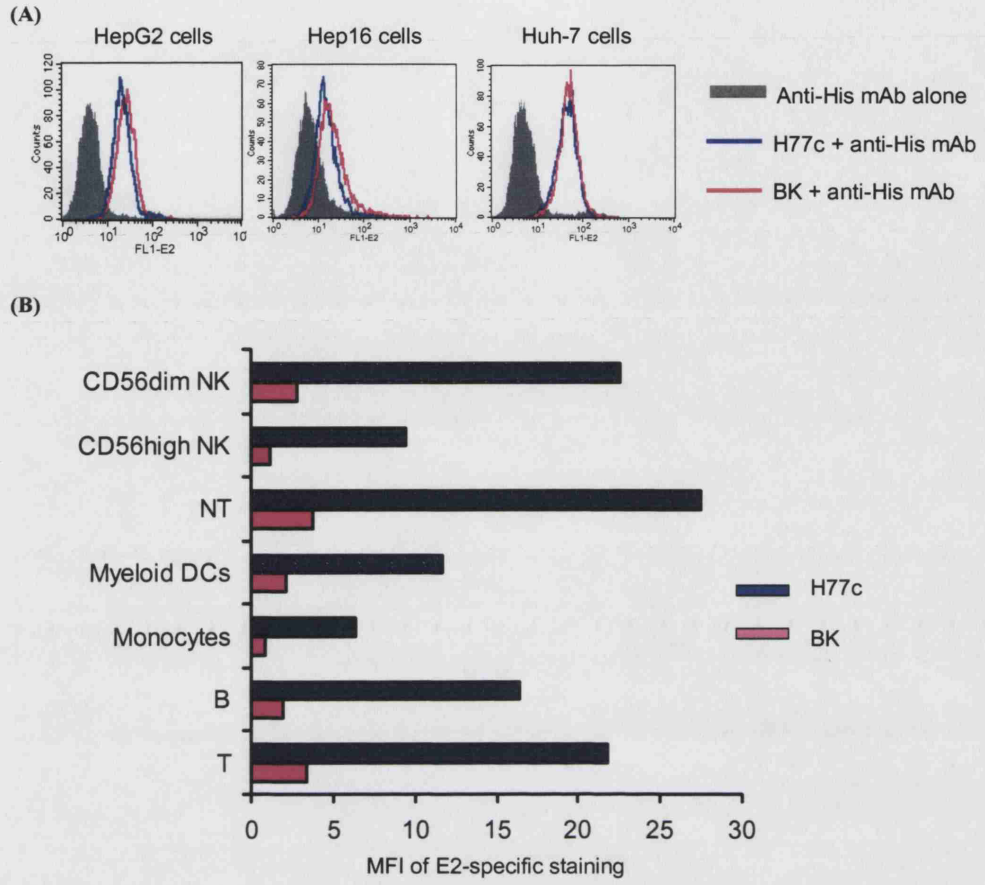


Figure 3.23. Binding of H77c and BK $E2_{660/661}$ to hepatocyte cell lines and PBMCs.

Cells were incubated with 70 μg of H77c or BK $E2_{661}$. E2 binding was detected using Alexa fluor 488-conjugated anti-Penta-His mAb. PBMC subsets were identified by co-staining with mAbs against surface markers as detailed in the legend to Fig. 3.1. (A) H77c and BK $E2_{661}$ binding to hepatocyte cell lines. In each panel, the grey shaded histogram represents the background binding of the anti-His mAb to the indicated cell lines in the absence of E2, and the line graphs represent staining of cells incubated with H77c (blue lines) or BK $E2_{661}$ (pink lines). The data shown are representative of results obtained in 3 independent experiments. (B) H77c and BK $E2_{660/661}$ binding to PBMC subsets. The results are expressed as the mean fluorescence intensity (MFI) of E2-specific staining of each subset after incubation of PBMCs with H77c (blue bars) or BK $E2_{661}$ (pink bars). The data shown are representative of findings made in 4 independent experiments using PBMCs isolated from different donors.

H77c and BK E2s also showed a very similar level of binding to Huh-7 and Hep16 cells, which express both SR-BI and CD81, suggesting that the levels of SR-BI and/or other receptors were sufficiently high not to limit E2 binding.

The binding of H77c and BK $E2_{660/661}$ to PBMCs was also compared, using the anti-Penta-His mAb to detect bound E2 (Fig. 3.23-B). Binding of BK $E2_{661}$ to all PBMC subsets was found to be much lower than that of H77c $E2_{660}$: just a low level of BK E2 binding to PBMCs was observed when saturating amounts of E2 protein were incubated with PBMCs. Similar results were obtained using two different batches of H77c and BK $E2_{660/661}$ proteins, confirming their reproducibility. The low level of BK binding to PBMC subsets is consistent with the finding that much of the binding of H77c E2 to PBMCs is mediated via interaction with CD81 (Fig. 3.17/3.18), and BK has a low CD81 binding ability (Fig. 3.22). Interestingly, although the level of BK $E2_{661}$ binding to PBMCs was very low, the pattern of binding of this protein to PBMC subsets appeared similar to that of H77c $E2_{660}$, i.e. the highest level of binding was observed to NK/NT/T subsets and lower binding was seen to monocytes, DCs and B cells.

3.2.13. Comparison of the binding of H77c, 1A14, BK, 1B12 E2s to PBMC subsets

The results above indicated that there was a marked difference in the binding of H77c and BK $E2_{660/661}$ to PBMCs. In order to investigate whether this difference represented a general characteristic of E2s from genotypes 1a and 1b, I produced soluble truncated $E2_{661}$ from another genotype 1a clone, 1A14, and another genotype 1b clone, 1B12, in parallel with H77c and BK $E2_{660/661}$. The 1A14 and 1B12 clones encode functional E2s, as retroviral pseudoparticles expressing HCV E1/E2 from these strains were shown to be infectious for Huh-7 cells (Lavillette *et al.*, 2005a).

To produce soluble E2 proteins, 293T cells were initially transfected with plasmids carrying the H77c, 1A14, 1B12 or BK $E2_{660/661}$ sequences using the GeneJuice method. However the yields of 1A14 and 1B12 $E2_{661}$ were considerably lower than those of H77c or BK $E2_{660/661}$. In order to improve the protein yield, 293FT cells were used to produce E2 proteins from the 1A14 and 1B12 clones. In addition, the calcium phosphate method was used to transfect the 293FT cells instead of the GeneJuice method. More than 99 % of 293FT cells were successfully transfected using the calcium phosphate method, which was determined by detecting cells expressing GFP by flow cytometry following transfection with an GFP-encoding plasmid. Higher concentrations of 1A14 and 1B12 $E2_{661}$ were obtained using this method, more similar to those of H77c or BK $E2_{660/661}$. The transfection efficiencies achieved using the GeneJuice and calcium phosphate methods were similar, but the protein yields were different. This could be because the number of plasmid copies transfected into each cell was higher using the calcium phosphate method than using the GeneJuice method. Batches of 1A14, 1B12, H77c and BK $E2_{660/661}$ were thus produced in parallel using the calcium phosphate transfection method for use in comparative binding studies.

After Ni^{++} column purification, the amount of monomeric E2 in each protein preparation (H77c, 1A14, 1B12 and BK) was determined by running the samples on a non-reducing SDS-PAGE gel followed by Western blotting using an anti-Penta-His mAb (Fig. 3.24). When the same concentration of E2 from each preparation was run on a gel, Western blotting showed that the H77c preparation contained more monomeric E2 than the other E2 preparations. In order to normalise the amount of monomeric E2 in the different preparations for use in binding studies, the relative monomer content in

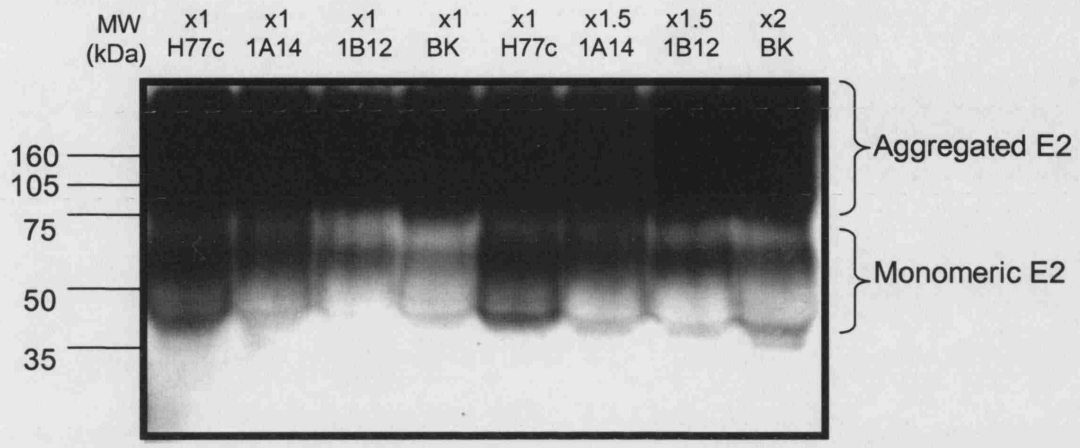


Figure 3.24. Normalisation of the monomeric E2 content of H77c, 1A14, 1B12 and BK E2 proteins by Western blotting.

Supernatants were harvested from 293FT cells transfected with plasmids expressing H77c, 1A14, 1B12, or BK $E2_{660/661}$, and E2 was purified using Ni^{++} columns. 30 μg (indicated as x1), 45 μg (indicated as x1.5), or 60 μg (indicated as x2) of E2 protein was run on SDS-PAGE gels together with molecular weight markers (MW), transferred and E2 was identified by Western blotting using an anti-Penta-His mAb. Bands representative of aggregated and monomeric forms of $E2_{660/661}$ are indicated.

each preparation was determined by quantifying the protein bands. It was found that the H77c preparation contained 1.5 times more monomers than the 1A14 and 1B12 E2 preparations and 2 times more monomers than the BK preparation. When "normalised" total quantities of each protein were run on a gel, Western blotting showed that more similar amounts of monomeric E2 were present in all four preparations (Fig. 3.24). In subsequent binding assays, the quantity of total protein of each preparation used was thus normalised so that equivalent amounts of monomeric E2 of each strain were always used.

Prior to their use in binding assays, 1A14 and 1B12 E2 proteins were characterised in parallel with H77c and BK E2s. Firstly, recognition of purified supernatant $E2_{660/661}$ from the H77c, 1A14, 1B12 and BK strains by a panel of anti-E2 mAbs was compared using a GNA lectin capture EIA (Fig. 3.25). H77c $E2_{660}$ was recognised by all mAbs tested including the conformation-dependent mAbs 9/86a, H53 and H50. 1A14 $E2_{661}$ was also recognised by the same panel of mAbs as H77c $E2_{660}$. However the genotype 1b E2 proteins 1B12 and BK were recognised only by anti-E2 mAbs directed against linear epitopes, and not by the conformation-dependent mAbs used in this study. This suggests that the mAbs directed against conformation-dependent epitope(s) are specific to genotype 1a.

Although both BK and 1B12 E2s were recognised by mAbs 3/11 and AP33, which are directed against linear epitope(s) in aa 412-423, 1B12 $E2_{661}$ was not recognised by other mAbs that did bind to BK, indicative of sequence differences between the two 1b clones.

Binding of H77c, 1A14, 1B12 and BK $E2_{660/661}$ to GNA lectin and to CD81 was also compared by EIA. Dose-dependent binding of purified supernatants from all four

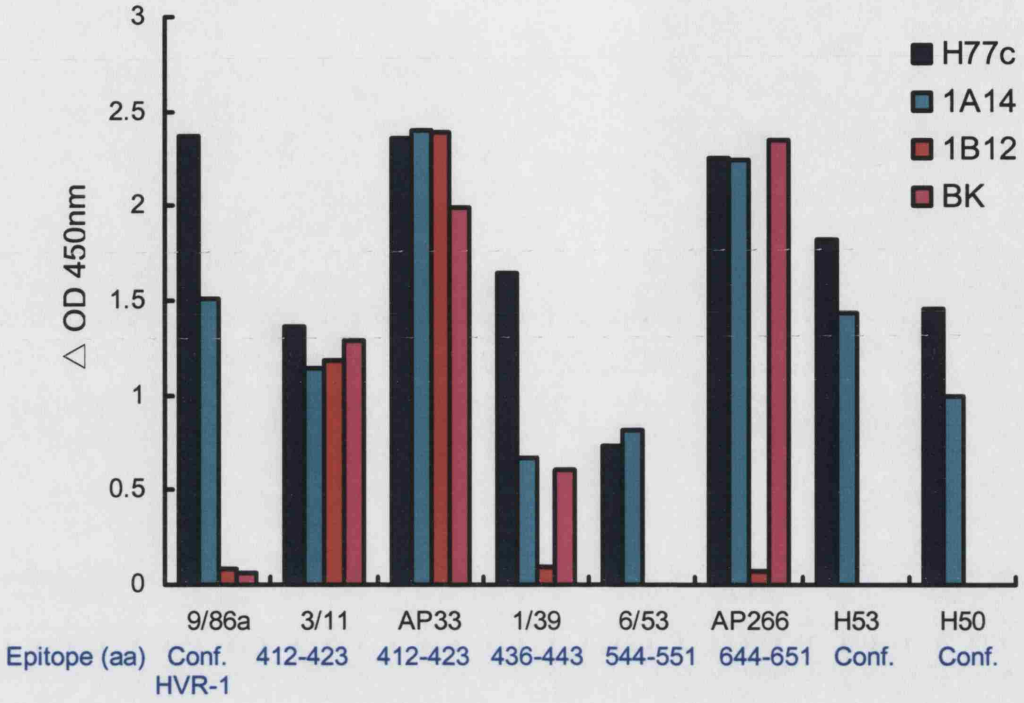


Figure 3.25. Recognition of supernatant forms of $E2_{660/661}$ derived from H77c, 1A14, 1B12 and BK strains by a panel of anti-E2 mAbs in a GNA lectin capture EIA.

Purified supernatant $E2_{660/661}$ derived from cells transfected with H77c (dark blue bars), 1A14 (light blue bars), 1B12 (red bars) or BK (pink bars) plasmids was immobilized in GNA lectin-coated wells. Bound $E2_{660/661}$ was detected using a panel of anti-E2 mAbs (3/11, 9/86a, 1/39, 6/53, AP266, AP33, H53, and H50) and a secondary mAb conjugated to HRP, followed by a colour reaction monitored at 450 nm. The results shown are Δ optical density (ΔOD) values, calculated as described in the legend to Fig. 3.11. Details of the epitopes recognised by each of the anti-E2 mAbs are indicated below the graph. MAb 9/86a recognises an epitope within hypervariable region-1 (HVR-1). MAbs recognising conformation dependent epitopes are denoted as Conf. Numbers represent the amino acid (aa) residues within E2 that contain the epitopes recognized by mAbs recognizing linear epitopes.

$E2_{660/661}$ proteins to GNA lectin was observed using an anti-Penta-His mAb (Fig. 3.26-A). H77c $E2_{660}$ bound to hCD81 in a dose-dependent fashion, but the other E2 proteins showed very little binding to hCD81 (Fig. 3.26-B). In previous experiments, I had found that anti-E2 mAbs provided a more sensitive means of detection of E2 proteins than the anti-Penta-His mAb. Therefore, the conformation-dependent anti-E2 mAb H53 (which recognises both H77c and 1A14 $E2_{660/661}$ (Fig. 3.25)) was used to confirm the relative levels of binding of H77c and 1A14 $E2_{660/661}$ to lectin and hCD81 in EIAs. Both H77c and 1A14 $E2_{660/661}$ bound to GNA lectin in a dose-dependent fashion (Fig. 3.26-C). In contrast, dose-dependent binding to hCD81 was only observed using H77c $E2_{660}$, and 1A14 $E2_{661}$ did not show detectable binding to hCD81 at the doses used in this assay. These results confirmed that not only genotype 1b E2s, but also some genotype 1a E2 proteins have a low affinity of binding to CD81.

Prior to characterising the binding of 1A14 and 1B12 $E2_{661}$ to PBMCs, their binding to hepatocyte cell lines was investigated. Binding of 1A14 and H77c $E2_{660/661}$ to hepatocyte cell lines was analysed using anti-E2 mAb H53 (Fig. 3.27-A), and binding of 1B12 $E2_{661}$ to hepatic cells was compared with that of H77c $E2_{660}$ using an anti-Penta-His mAb (Fig. 3.27-B). Both 1A14 and 1B12 $E2_{661}$ proteins were found to bind to all hepatocyte cell lines tested. The ability of 1A14 and 1B12 $E2_{661}$ to bind to HepG2 cells ($CD81^-$ $SR\text{-}BI^+$) indicates that although these $E2_{661}$ proteins have a low affinity of binding to CD81, they can bind to SR-BI and/or other receptor(s) that are expressed on hepatocyte cell lines. The level of binding of 1A14 and H77c $E2_{660/661}$ proteins to HepG2 cells was very similar, but the level of 1B12 $E2_{661}$ binding to HepG2 cells was higher than that of H77c $E2_{660}$. It is thus plausible that 1B12 $E2_{661}$ has a higher binding affinity to SR-BI and/or other receptor(s) than H77c $E2_{660}$. In support of this, Lavillette *et al.* reported that each HCV strain has different capacity to utilise SR-BI for its

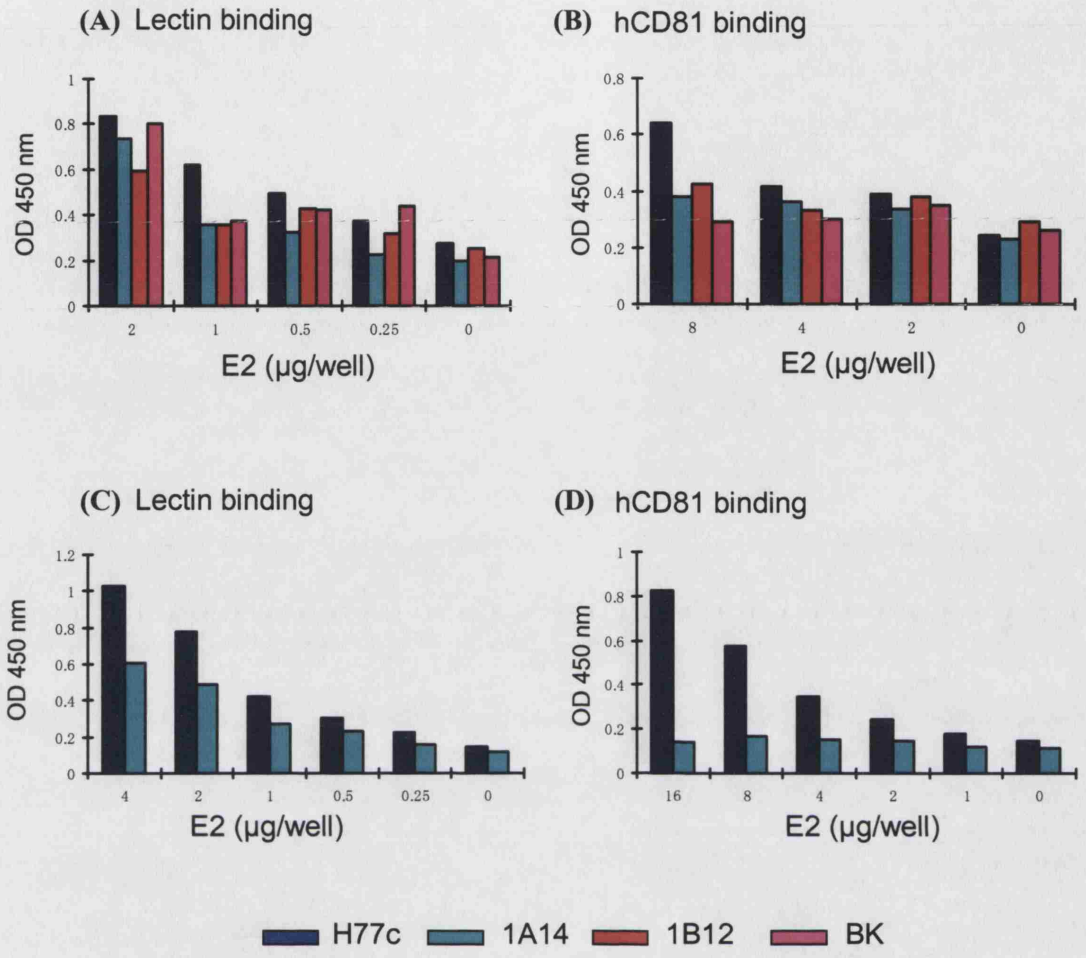


Figure 3.26. Binding of $E2_{660/661}$ from H77c, 1A14, 1B12 and BK strains to GNA lectin and hCD81 as assessed by EIA.

The binding of purified supernatant H77c, 1A14, 1B12 and BK $E2_{660/661}$ to GNA lectin and human CD81 (hCD81) was measured by EIA. The indicated concentrations of H77c (dark blue bars), 1A14 (light blue bars), 1B12 (red bars) or BK $E2_{661}$ (pink bars) were incubated in plates coated with GNA lectin ((A) and (C)) or hCD81 ((B) and (D)). Bound $E2_{660/661}$ was detected using an anti-Penta-His mAb ((A) and (B)) or mAb H53 ((C) and (D)) and a HRP-conjugated secondary mAb, followed by a colour reaction monitored at 450 nm. The results shown are the optical density (OD) values obtained for wells to which different concentrations of E2 were added.

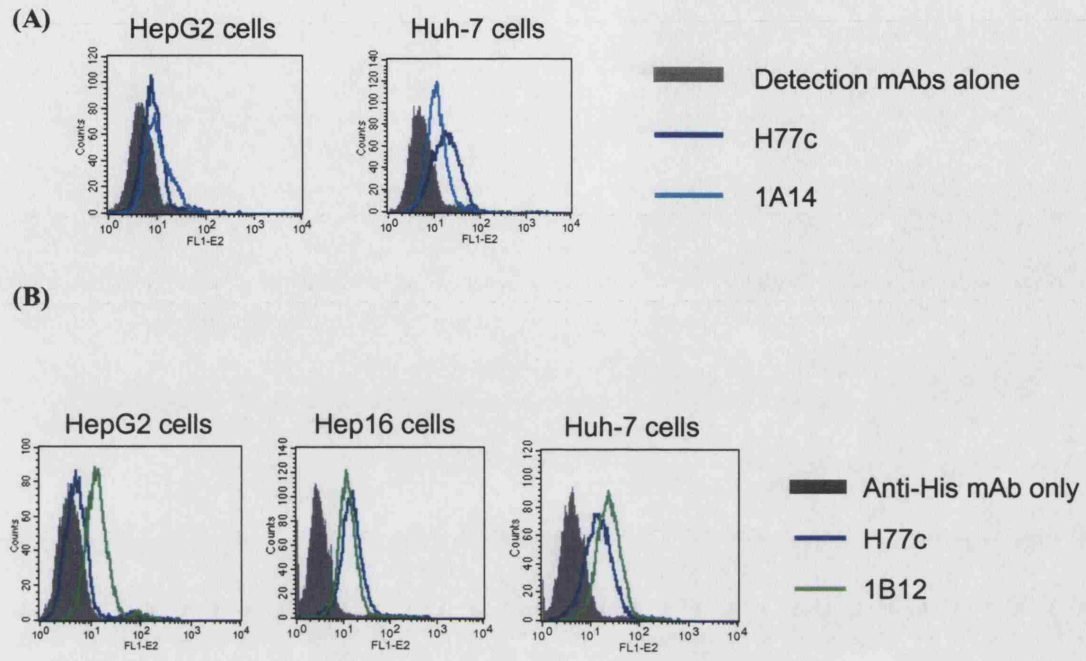


Figure 3.27. Binding of $E2_{660/661}$ from H77c, 1A14 and 1B12 strains to hepatocyte cell lines.

Hepatocyte cell lines were incubated with 70 μg of H77c or 105 μg of 1A14 or 1B12 $E2_{661}$. Binding of H77c and 1A14 $E2_{660/661}$ was detected using anti-E2 mAb H53 and a FITC-conjugated secondary mAb (A). Binding of H77c and 1B12 $E2_{661}$ was detected using an Alexa Fluor 488-conjugated anti-Penta-His mAb (B). In each panel, the grey shaded histogram represents the background binding of detection mAb(s) to the indicated cell lines in the absence of E2, and the line graphs represent staining of cells incubated with H77c (dark blue lines), 1A14 $E2_{661}$ (light blue lines) or 1B12 $E2_{661}$ (green lines). The data on E2 binding to HepG2 cells are from a single experiment and the rest of the data shown are representative of findings made in 2 independent experiments.

infection (Lavillette *et al.*, 2005a). However, more experiments are required to confirm this result.

Further experiments went on to compare the binding of H77c and 1A14 $E2_{660/661}$ to PBMCs, using anti-E2 mAb H53 to detect bound E2 (Fig. 3.28). 1A14 $E2_{661}$ was found to bind to all PBMC subsets much less well than H77c $E2_{660}$. As observed with BK E2, the pattern of binding of 1A14 E2 protein to different PBMC subsets was similar to that of H77c $E2_{660}$, i.e. the highest binding was seen to NK/NT/T subsets and binding to monocytes, DCs and B cells was lower. These observations were confirmed in experiments using two independently-prepared batches of 1A14 and H77c $E2_{660/661}$, one which I produced, and the other which was provided by Dr Tim Hickling (Division of Microbiology and Infectious Diseases, University of Nottingham, UK).

The binding of 1B12, 1A14, H77c and BK $E2_{660/661}$ to PBMC subsets was then compared in experiments using an anti-Penta His mAb to detect bound E2 (Fig. 3.29). The binding of 1A14, 1B12 and BK $E2_{661}$ to all PBMC subsets was found to be much lower than that of H77c $E2_{660}$. This was not unexpected, given the low level of binding of 1A14, 1B12 and BK $E2_{661}$ to CD81 observed by EIA (Fig. 3.26).

3.2.14. Binding of VLPs to PBMCs

HCV possesses two envelope glycoproteins, E1 and E2. When expressed *in vitro*, E1 and E2 form heterodimers, which are assumed to be the native form of the virion surface glycoprotein (Flint & McKeating, 1999). Although soluble truncated E2 proteins are thought to be folded in a manner comparable to that of E2 in E1E2 complexes, it is possible that there may be differences in binding between soluble E2 proteins and the native viral form of E2. Three series of experiments were thus carried

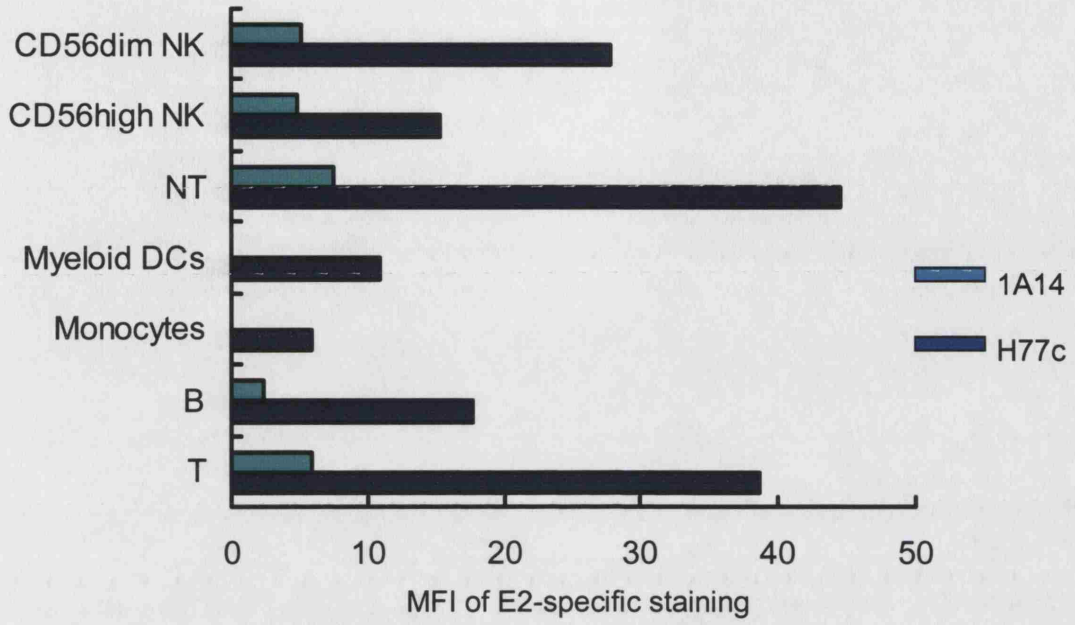


Figure 3.28. Binding of $E2_{660/661}$ from the H77c and 1A14 strains to PBMC subsets.

PBMCs were incubated with 70 μg of H77c or 105 μg of 1A14 $E2_{661}$ and bound E2 was detected using anti-E2 mAb H53 and a FITC-conjugated secondary mAb. PBMC subsets were identified by co-staining with mAbs against surface markers as detailed in the legend to Fig. 3.1. The results are expressed as the mean fluorescence intensity (MFI) of E2-specific staining (calculated as described in the legend to Fig. 3.13) of each subset after incubation of PBMCs with H77c (dark blue bars) or 1A14 $E2_{661}$ (light blue bars). The data shown are representative of 2 independent experiments using PBMCs isolated from different donors.

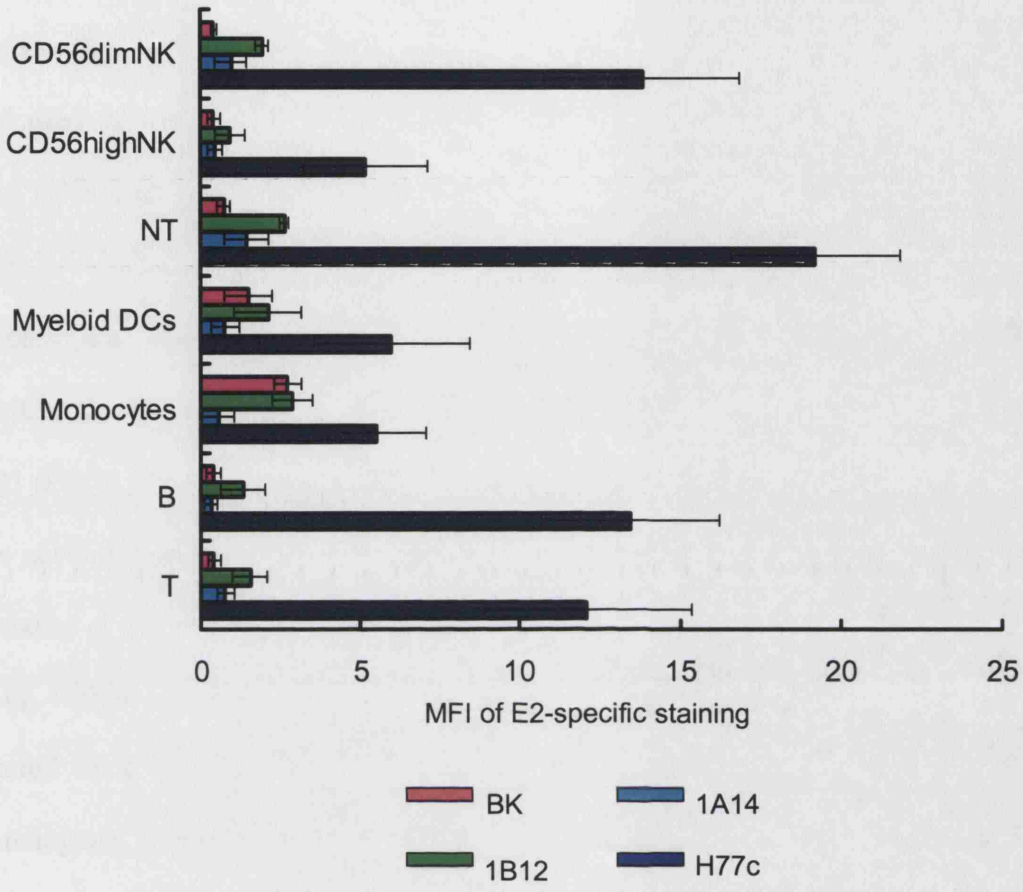


Figure 3.29. Binding of $E2_{660/661}$ from H77c, 1A14, 1B12 and BK strains to PBMC subsets.

PBMCs were incubated with 70 μg of H77c, 105 μg of 1A14 or 1B12, or 140 μg of BK $E2_{661}$ and bound E2 was detected using an Alexa Fluor 488-conjugated anti-Penta-His mAb. PBMC subsets were identified by co-staining with combinations of mAbs against surface markers as detailed in the legend to Fig. 3.1. The results shown are the mean fluorescence intensity (MFI) of E2-specific staining of each subset (calculated as described in the legend to Fig. 3.13) after incubation of PBMCs with the indicated E2 proteins. The data shown are the mean of results obtained in 3 independent experiments; the error bars indicate standard error above the mean.

out to investigate the binding of more "native" forms of E2 - expressed as heterodimers with E1 on the surface of particles - to PBMCs. The first employed HCV VLPs produced in insect cells using a baculovirus expression system. Two forms of VLPs were used in this project: VLPs expressing E1E2 heterodimers from infectious HCV clone H77c and VLPs expressing E1 from H77c and E2 from C3. These were provided by Dr Arvind Patel (MRC Virology Unit, Institute of Virology, Glasgow, UK). In order to determine whether VLPs expressing HCV E1E2 heterodimers could interact with PBMCs, the binding of these VLPs to PBMCs was investigated using anti-E2 mAb AP33 (Fig. 3.30). It has been reported that mAb AP33 recognises H77c VLPs and that it does not interfere with the interaction between VLPs and CD81 (Clayton *et al.*, 2002; Owsianka *et al.*, 2001). As shown in Fig. 3.30-B, no VLP binding was detected to cells falling within a lymphocyte gate (R1 in Fig. 3.30-A). Further, no VLP binding was detected to T cells, NK cells, B cells, monocytes, myeloid DCs, granulocytes or plasmacytoid DCs (data not shown). However, as shown in Fig. 3.30-C, VLP binding was detected to cells falling within the R2 gate in Fig. 3.30-A. 50 % of R2 gated cells were bound by both H77c and C3 VLPs. The cells within the R2 gate were bigger and more granular than lymphocytes. Most $CD14^{high}$ monocytes were found in the R2 gated region, but no VLP binding was detected to purified $CD14^{+}$ monocytes (data not shown). Thus it is unlikely that cells in the R2 gated region with which VLPs were interacted were monocytes. They may potentially have been neutrophils or eosinophils. It is unclear whether the VLP binding to cells in the R2 gate was specific or not: it was not saturable at the VLP concentrations tested (data not shown).

VLP binding to monocyte-derived DCs was also analysed (Fig. 3.30-D). Both H77c and C3 VLPs were found to bind to these cells at the same level. Given that C3 $E2_{660}$ does not bind efficiently to CD81 (Patel *et al.*, 2000), this suggests that the interaction of

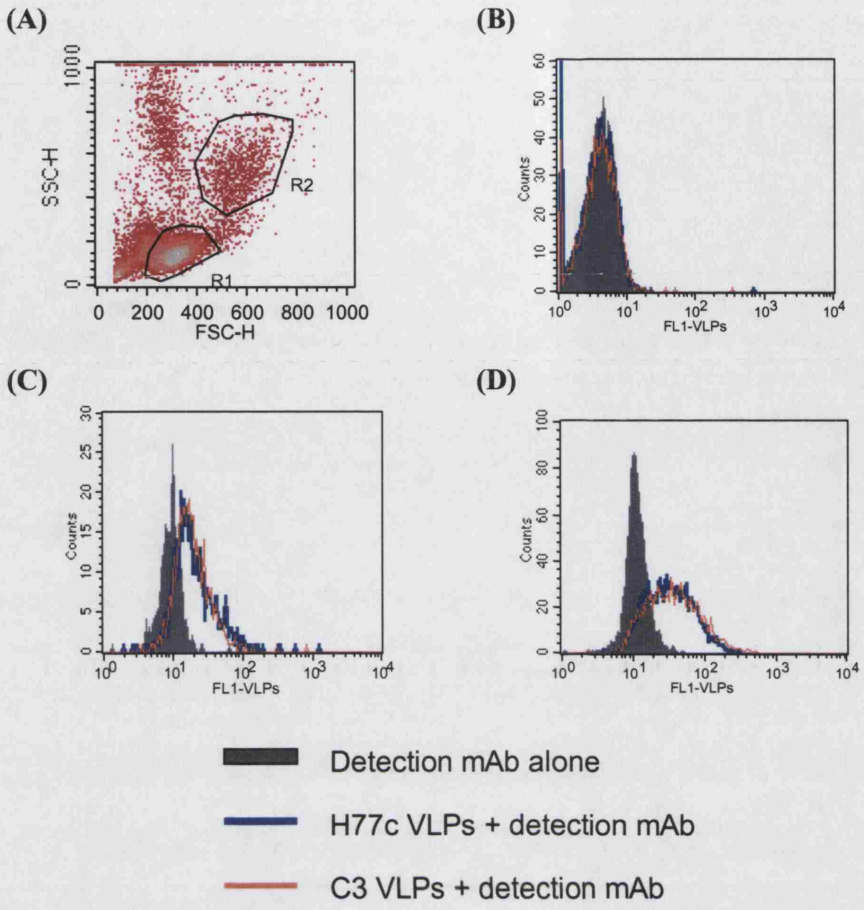


Figure 3.30. Binding of VLPs to PBMCs and monocyte-derived DCs.

Virus like particles (VLPs) expressing E1E2 heterodimers from H77c (H77c VLPs) or C3 (C3 VLPs) were incubated with PBMCs or monocyte-derived DCs at 4 °C, then VLP binding to the cells was detected using fluorescent-conjugated anti-E2 mAb AP33. (A) Dotplot showing the forward scatter (FSC) and side scatter (SSC) profile of PBMCs, and illustrating the regions gated for analysis of VLP binding. Cells in R1 are small mononuclear cells (largely lymphocytes), and cells in R2 are larger, more granular cells (largely monocytes). (B) VLP binding to cells in region R1. (C) VLP binding to cells in region R2. (D) VLP binding to monocyte-derived DCs. In panels (B)-(D), the grey histograms show the background binding of the detection mAb in the absence of VLPs and the line graphs represent the staining of gated cells after incubation of cells with H77c VLPs and the detection mAb (blue lines) or C3 VLPs and the detection mAb (orange lines). The results shown are representative of 10 independent experiments using PBMCs isolated from different donors.

VLPs with monocyte-derived DCs was not mediated via CD81. Further, pre-incubation of VLPs with anti-E2 mAb H33 (which can block the interaction between E2 and CD81 (Patel *et al.*, 2000), and is reported to recognise H77c VLPs (Wellnitz *et al.*, 2002)) prior to addition to monocyte-derived DCs did not block the binding of H77c or C3 VLPs to these cells (data not shown). It is not known whether mAb H33 can block the interaction of E2 proteins with other receptors such as SR-BI (although the results in Fig. 3.17 suggest that it is unlikely to do so). It thus remains unclear whether this binding of VLPs to monocyte-derived DCs was non-specific binding, or was specific binding mediated through receptors other than CD81. Had time permitted, it would have been interesting to follow up these observations further.

3.2.15. Binding to and infection of PBMCs of HCV pseudotyped retroviral particles

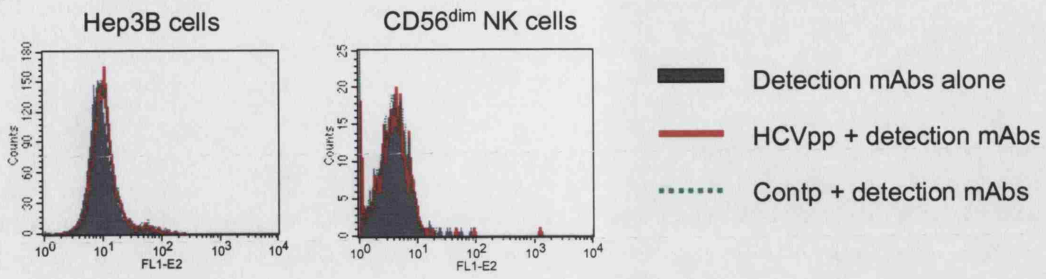
A second reagent used to investigate whether there are any differences in the binding of soluble truncated E2 and E2 in the form of E1E2 heterodimers expressed on particles to PBMCs was retroviral pseudoparticles expressing HCV E1E2. Two types of pseudoparticles were used in this study: HCV pseudotyped particles (HCVpp) expressing E1E2 heterodimers from the infectious HCV clone H77c and control particles without HCV glycoproteins (Contp). These particles were assembled on cores from MLV, and were provided by Dr Anne Goffard, Dr Cécile Voisset and Dr Jean Dubuisson (Institute de Biologie de Lille & Institut Pasteur de Lille, Lille, France).

Initial experiments examined the binding of HCVpp to hepatocyte cell lines and PBMCs. HCVpp were incubated with cells, and HCVpp binding was detected using conformation-dependent anti-E2 mAbs H48, H33 or H53 which have been shown to recognise H77c HCVpp (Op De Beeck *et al.*, 2004). No binding of HCVpp could be

detected to any of the cell types tested (Hep3B, Huh-7, Hep16 and Molt-4 cell lines and PBMC subsets including NK cells, T cells, B cells, DCs and monocytes). Examples of the data are shown in Fig. 3.31-A. H77c HCVpp have been shown to infect hepatocyte cell lines (Bartosch *et al.*, 2003a), thus must bind to these cells: but the level of binding was not high enough to detect by mAb staining and flow cytometry.

Infectivity assays represent a more sensitive method of investigating the interaction of HCVpp with target cells. Attempts were thus also made to address the infectivity of HCVpp for PBMC subsets. The HCVpp provided carried a luciferase reporter gene, allowing infection of cells by HCVpp to be evaluated by assessing luciferase expression. In infectivity assays, test cells (Hep3B cells, monocyte-derived DCs, monocytes, T cells and B cells) were incubated with either HCVpp or Contp for 3 hours, washed and cultured for 72 hours to allow the expression of luciferase genes. One of the drawbacks to the use of MLV as a vector is its inability to transduce non-dividing target cells. In order to induce the proliferation of T and B cells, they were stimulated with PHA or Ig beads and IL-4 respectively during the 72 hour culture period. Monocyte-derived DCs and monocytes were activated with LPS, to induce activation. The stimulation of these cells was confirmed by the detection of activation/maturation markers on their surface (data not shown). Luciferase expression was observed in lysates of Hep3B cells that had been incubated with HCVpp but not with Contp or control cells, indicating that HCVpp infected Hep3B cells (Fig. 3.31-B). However, no infection of monocyte-derived DCs, monocytes, T cells or B cells could be detected. As considered in the discussion, this may well have reflected inability of the MLV-based pseudoparticles to transduce human PBMCs. No further experiments were thus carried out using HCVpp.

(A)


Hep3B cells
CD56dim NK cells
Counts
FL1-E2
Detection mAbs alone
HCVpp + detection mAbs
Contp + detection mAbs

(B)

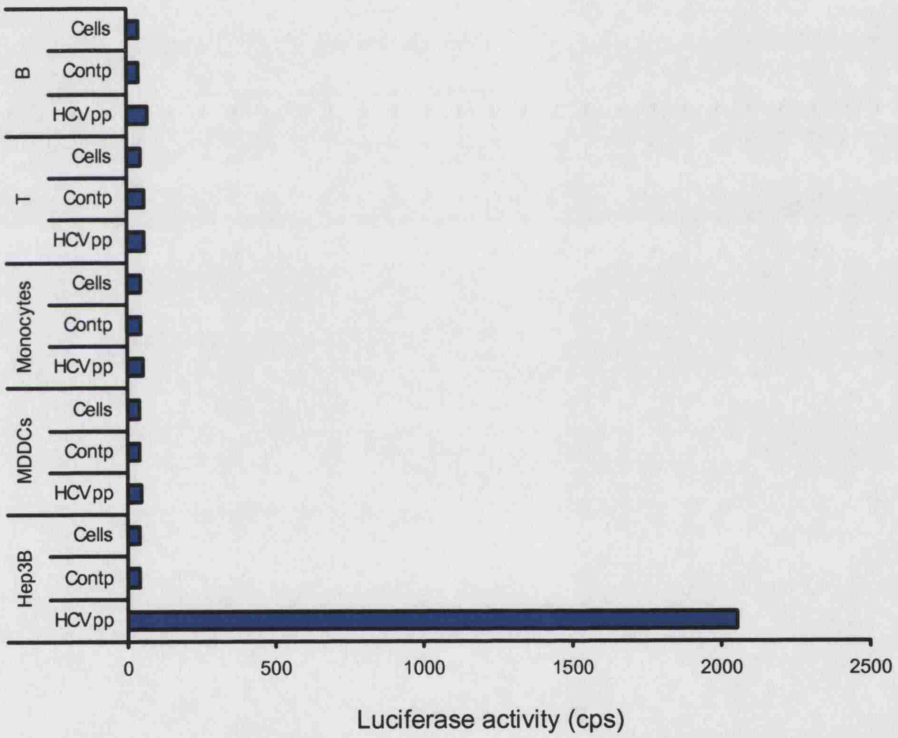

B
T
Monocytes
MDDCs
Hep3B
Cells
Contp
HCVpp
0
500
1000
1500
2000
2500
Luciferase activity (cps)

Figure 3.31. Binding to and infectivity of HCV pseudoparticles for Hep3B cells, PBMCs and monocyte-derived DCs.

HCV pseudotyped retroviral particles (HCVpp) expressing E1E2 heterodimers from the H77c strain or control particles (Contp) which did not express E1E2 were used. (A) Binding of HCVpp to Hep3B or $CD56^{dim}$ NK cells. Hep3B cells or PBMCs were incubated with HCVpp or Contp at 4 °C, then were stained with anti-E2 mAb H48 and a FITC-conjugated secondary mAb. PBMCs were co-stained with mAbs against CD3 and CD56 to enable identification of $CD56^{dim}$ NK cells. The binding of HCVpp was analysed by flow cytometry. In each panel, the grey shaded histogram represents the background staining of the indicated cells with the detection mAbs, and the line graphs represent the staining of cells incubated with HCVpp (red lines) or Contp (green dotted lines). The results shown are representative of findings made in 3 independent experiments using PBMCs from different donors. (B) Infectivity of HCVpp for Hep3B cells and PBMC subsets. Hep3B cells, monocyte-derived DCs, purified monocytes, T cells and B cells were treated in different ways before infection as described in the Materials and Methods, then cells were incubated with Contp or HCVpp for 3 hours. After washing to remove unbound particles, the cells were incubated for 72 hours to allow the luciferase gene to be expressed. Cell lysates were then prepared from cells incubated without particles (Cells) and cells infected with Contp (Contp) or with HCVpp (HCVpp), and luciferase activity was measured in the lysates. The results shown are the luciferase activity in each sample, expressed as counts per second (cps). The results shown are representative of findings made in 2 independent experiments.

3.2.16. Binding of HCV virions to PBMCs

A final series of experiments examined the binding of HCV virions to PBMC subsets. Lipoprotein and immunoglobulin free-HCV virions isolated from the serum of a patient infected with genotype 1a HCV were provided by Dr Christian Schuettler (Institute of Medical Virology, Justus-Liebig University, Giessen, Germany). I determined whether these HCV virions bound to PBMC, and whether the pattern of virion binding to different PBMC subsets was similar to that of recombinant soluble E2 proteins. HCV virions were incubated with PBMCs, and virion binding was detected using mAb H53 (Fig. 3.32). A low level of virion binding was observed to $CD56^{dim}$ NK cells and NT cells, both of which express high levels of CD81. Virion binding was not detected to any other PBMC subsets. It is unclear whether virions cannot bind to other cell subsets, or whether the binding level was too low to be detected by mAb staining or flow cytometry. The fact that virion binding was not observed to all PBMC subsets suggested that the interaction seen was probably not non-specific. Further, the fact that those PBMC subsets to which virion binding was detected were those expressing the highest levels of CD81 would be consistent with CD81 playing a role in virion binding to cells. Unfortunately, however, only very limited quantities of virions were available, so it was not possible to carry out blocking studies to explore these issues. Nevertheless, the results obtained show that HCV genotype 1a virions bind to at least certain PBMC subsets expressing high levels of CD81.

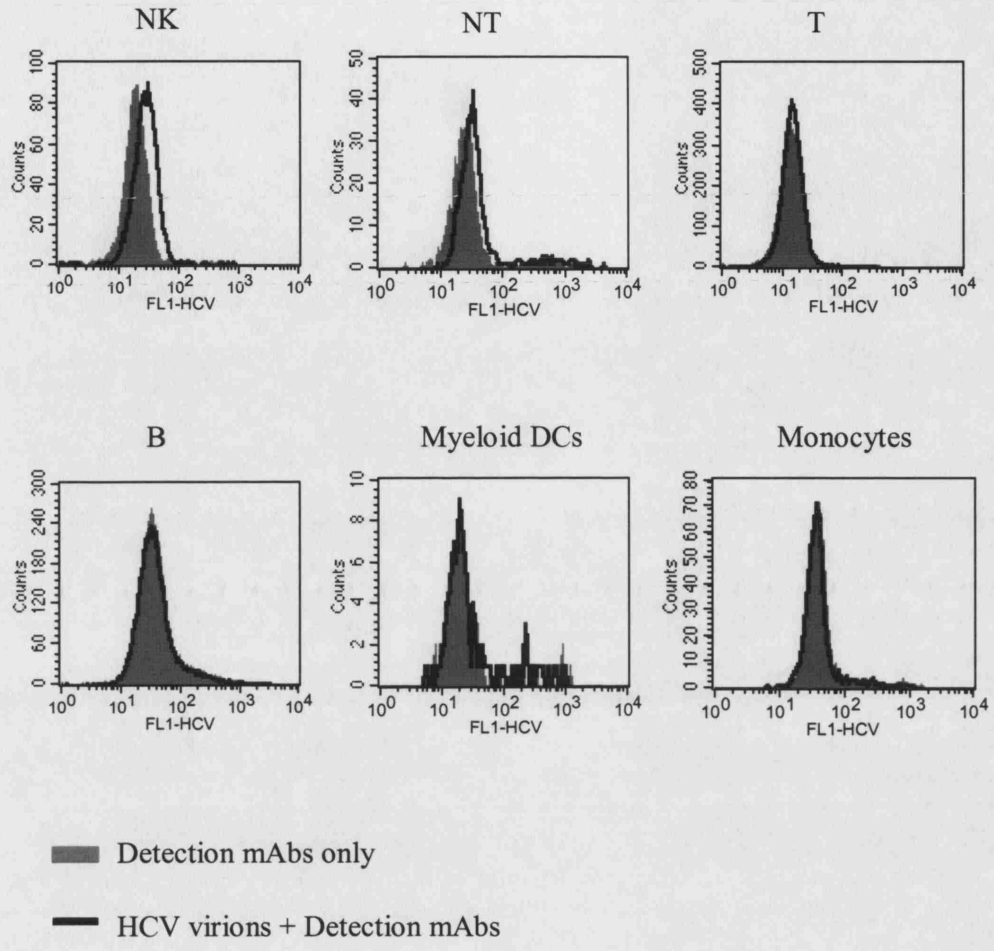


Figure 3.32. Binding of HCV virions to PBMC subsets.

PBMCs were incubated at 4 °C with HCV virions isolated from the serum of a patient infected with a genotype 1a HCV, then HCV particle binding to the cells was detected using anti-E2 mAb H53 and a FITC-conjugated secondary mAb. PBMC subsets were identified by co-staining with mAbs against distinguishing surface markers. The grey shaded histogram in each panel represents staining of cells of the indicated subset with the detection mAbs only, and the black line shows staining of cells incubated with HCV virions. The results shown are from one representative experiment of 2 carried out.

3.3. Discussion

PBMCs have been suggested as extrahepatic replication sites for HCV. Although the interaction between E2 proteins and receptors on hepatocytes has been intensively investigated, no studies have characterised the interaction of E2 proteins with *ex vivo* PBMC subsets. In this chapter, the binding of different forms of E2 glycoproteins to PBMC subsets was investigated in parallel with the expression of CD81 and SR-BI on these cells.

CD81 is expressed on most cells except for red blood cells and platelets (Engel & Tedder, 1994), with expression levels varying in different human tissues (Levy *et al.*, 1998). All PBMC subsets investigated in this study were found to express CD81; however, there was variations in the level of CD81 expression detected on different subsets. NK, NT and T cells expressed high levels of CD81, whereas DCs, monocytes, granulocytes and B cells expressed lower levels of this molecule. These results agreed with previous studies that addressed CD81 expression on some of the PBMC subsets analysed here (Tohami *et al.*, 2004; Tsuji *et al.*, 2002). CD81, a member of tetraspanin superfamily, can associate with various membrane proteins on different cell types. Thus, it cannot be excluded that on those cells where high CD81 expression was not seen, CD81 is complexed with other cell surface proteins in such a way that the binding site of the anti-CD81 mAb used in this study is not readily accessible. This possibility could be tested by carrying out immunoprecipitation for CD81 and associated proteins from each subset after treatment with different detergents. Alternatively, it is possible that CD81 is differentially expressed due to its functions in different PBMC subsets. CD81 is known to co-stimulate T and B cell activation (Carter & Fearon, 1992; Todd *et al.*,

1996). Interestingly, activated/memory B and T cells were observed to express higher levels of CD81 than naïve/resting cells, although the differences were not statistically significant. These results suggest a possible role for CD81 on antigen-experienced cells in allowing them to respond to lower concentrations of antigen and hence react more quickly to the presence of infection than naïve cells.

SR-BI has previously been shown to be expressed on monocytes and macrophages (Buechler *et al.*, 1999); here, I also detected its expression on plasmacytoid DCs, myeloid DCs and monocyte-derived DCs. In addition to facilitating the cellular uptake of cholesterol, SR-BI also functions as a pattern-recognition receptor that plays a critical role in innate immunity by recognising conserved microbial components (Pearson, 1996; Vishnyakova *et al.*, 2003). It is speculated that SR-BI expression on both monocytes and DCs may contribute to host defence.

This study also showed that SR-BI was much more readily detected on the hepatocyte cell lines than on monocytes and DCs, although direct comparison of the SR-BI expression on the hepatocyte cell lines and PBMC subsets was not possible. It is plausible that the level of expression of SR-BI on PBMCs may limit susceptibility of HCV to their infection to haematopoietic cells.

The main objective of the work in this chapter was to characterise the binding of the HCV E2 glycoproteins to PBMC subsets and investigate the roles of CD81, SR-BI and other receptor(s) in mediating the interaction between E2 and different cell subsets.

I initially planned to produce both cell-associated and supernatant soluble $E2_{660}$ since it has been suggested that cell-associated $E2_{661}$ may be antigenically more similar to native E2 than supernatant $E2_{661}$ (Flint *et al.*, 2000; Heile *et al.*, 2000). Preparations of supernatant $E2_{660}$ contained both aggregated and monomeric E2 of the expected MW,

which was successfully purified by Ni^{++} column chromatography. However, problems were encountered with the production of cell-associated $E2_{660}$. The yield was lower than expected, and Western blotting revealed that the preparations of cell-associated $E2_{660}$ did not contain aggregates of E2, and that the monomeric $E2_{660}$ present had a lower MW than observed in previous studies (Flint *et al.*, 2000). This suggests that the method for E2 extraction from cells may not have been optimal, with much of the cell-associated E2 being lost or partially degraded. Attempts were made to improve the extraction method, but without success. In addition, the cell-associated $E2_{660}$ that was produced could not be purified on Ni^{++} columns. This may have been because the histidine residues on the cell-associated E2 protein were not exposed in the same way as on the supernatant E2. In line with this, the cell-associated H77c $E2_{660}$ did not appear to be recognised by an anti-Penta-His mAb as well as the supernatant $E2_{660}$. Had time permitted, the method used for extracting cell-associated E2 could have been refined, and techniques for its purification optimised (perhaps utilising a larger column with a higher concentration of Ni^{++}; or using an affinity column made with an anti-E2 mAb for E2 purification). Since supernatant E2 proteins were successfully produced and purified, further experiments used this material to investigate the interaction of E2 with PBMC subsets.

All supernatant soluble truncated E2 proteins (H77c, C3, Gla, BK, 1A14 and 1B12 HCV clones) contained aggregates in the preparations; in particular almost all of the Gla $E2_{660}$ was aggregated. It is not clear what makes Gla $E2_{660}$ more aggregated and misfolded. However, the replacement of aa 524-660 of the Gla E2 sequence with that of H77c $E2_{660}$ makes C3 $E2_{660}$ properly folded and less aggregated. The possibility of separating monomeric and aggregated forms of E2 so that binding assays could be

carried out using highly concentrated monomeric E2 was explored. However, HPLC using a TSK column failed to separate them (Fig. 3.10); further, it has been observed that fractions containing mainly monomeric E2 tend to become aggregated after storing at 4 °C (unpublished observation of Dr Maria Montoya, the Edward Jenner Institute for Vaccine Research, UK). Therefore, $E2_{660/661}$ preparations containing both aggregated and monomeric material were used in binding assays, but where possible, batches of different E2s were normalised to contain similar levels of monomeric E2. In experiments using Gla $E2_{660}$, the same quantity of total protein was used of all E2 studied.

In experiments where the conformation-dependent anti-E2 mAb H53, which recognises only monomeric E2 (Flint *et al.*, 2000), was used to detect bound E2, all the E2 binding observed should have been that of monomeric E2. Conversely, in experiments where an anti-Penta-His mAb was used to detect bound E2, the binding observed could have been that of both monomers and aggregates. Although the aggregated E2 protein does not bind to CD81 (Flint *et al.*, 2000), it is unclear whether it can bind to other receptors. It is possible that aggregated E2 protein may bind non-specifically to cells, or like monomeric E2, may bind to receptors other than CD81 e.g. GAGs or SR-BI (potentially competing with monomeric E2 for binding to these receptors). That aggregated E2 may have been able to bind to some cell surface receptors is supported by the observation that the binding of BK $E2_{661}$ to different PBMC subsets (detected using an anti-Penta-His mAb) shown in Fig. 3.23 and Fig. 3.29 appeared to be slightly different; in the former experiment, the pattern of BK E2 binding was similar to that of H77c $E2_{660}$, whereas in the latter experiment, the PBMC subset to which genotype 1b BK and 1B12 $E2_{661}$ proteins bound at the highest level was monocytes. The BK $E2_{661}$ preparations used in the former experiment contained relatively similar amounts of monomeric E2 to

the H77c E2 preparations, as shown by Western blotting (Fig. 3.21). Conversely, the amount of monomeric E2 in the BK and 1B12 preparations used in the latter experiment was considerably lower than that in the parallel H77c preparation, as shown by Western blotting (Fig. 3.24). The presence of a higher proportion of aggregates in the genotype 1b E2 preparations used in the latter binding assays (Fig. 3.29) might have contributed to the relatively higher binding of these E2s to APCs such as monocytes and DCs. These cells are bigger than lymphocytes and express various lectin receptors, thus they may be more prone to bind aggregated proteins. Alternatively, the aggregates might have bound to receptors such as SR-BI expressed on APCs. Had time permitted, additional experiments could have been performed with HPLC-separated fractions of H77c $E2_{660}$ containing different ratios of aggregated:monomeric E2 to address these issues further.

When studying the functions of E2, it is important to consider that soluble truncated E2 proteins differ from full-length E2 or E1E2 heterodimers expressed on the virion surface both in recognition by anti-E2 mAbs (Clayton *et al.*, 2002), and in their affinity of binding to putative receptors (Cocquerel *et al.*, 2003; Triyatni *et al.*, 2002a; Zhang *et al.*, 2004).

I carried out a small number of experiments using HCV virions purified from the serum of a patient infected with genotype 1a HCV. Native virions are the ideal material with which to investigate the nature of HCV binding. However, their use is hampered by limitations on availability; *in vitro* culture systems supporting the production of HCV particles have only recently begun to be developed and it is rare to find patients with a sufficiently high concentration of virus in the serum to allow virion purification. Thus two types of surrogate particles were also used in the binding assay.

The first type was VLPs produced in insect cells using a baculovirus expression system. Although both H77c soluble truncated $E2_{660}$ and H77c VLPs were previously shown to bind to CD81 in a CD81 capture EIA (Clayton *et al.*, 2002), the affinities of these two forms of E2 for CD81 may be different. Several reports found that the binding of H77c VLPs to hepatocyte cell lines (Huh-7 and HepG2 cells) and the Molt-4 T cell line was not mediated by CD81 (Steinmann *et al.*, 2004; Triyatni *et al.*, 2002b; Wellnitz *et al.*, 2002). Because H77c $E2_{660}$ binding to PBMCs was mainly mediated through CD81, the failure to detect VLP binding to lymphocytes in this study could have been due to the absence or low level expression on lymphocytes of the alternative receptor(s) that mediate VLP binding to hepatocyte cell lines. Alternatively, it is possible that VLP binding to lymphocytes was just too low to be detected by mAb staining and flow cytometry.

Although VLPs did not exhibit detectable binding to lymphocytes, H77c and C3 VLPs were found to bind to monocyte-derived DCs (Fig. 3.30). Given that soluble truncated C3 $E2_{660}$ binds very weakly to PBMCs, whilst it binds at higher levels to HepG2 cells and monocyte-derived DCs, it is possible that SR-BI or other receptor(s) expressed on HepG2 cells or monocyte-derived DCs mediated the binding of H77c and C3 VLPs. Ludwig *et al.* reported that VLPs can be internalised into monocyte-derived DCs in a DC-SIGN dependent manner (Ludwig *et al.*, 2004). Thus it is plausible that H77c and C3 VLP binding to these cells may be mediated through DC-SIGN that is highly expressed on immature monocyte-derived DCs (Baribaud *et al.*, 2002; Geijtenbeek *et al.*, 2000c).

Alternatively, the VLP binding I observed could have been non-specific. My collaborators in Glasgow investigated the binding of the batch of VLP that I used in the experiments to CHO cells that were induced to express human SR-BI, and obtained

inconsistent results. In some experiments, they detected the binding of both H77c VLPs and control VLPs (VLPs without HCV E1E2) to control CHO cells, suggesting the presence of non-specific interaction. Other studies have also found inconsistencies in the results of experiments carried out with VLPs (Bartosch *et al.*, 2003a; Baumert *et al.*, 1998). Thus although VLPs may be a useful tool to understand the morphology and antigenicity of HCV particles, they may not be the best tool for investigation of the HCV attachment/entry process.

The second reagent used was retroviral pseudoparticles expressing HCV E1E2. As shown in Fig. 3.31-A, binding of HCVpp was not detected to Hep3B cells or any PBMC subsets. Since HCVpp successfully infected Hep3B cells, as shown in Fig. 3.31-B, the failure to detect HCVpp binding to Hep3B cells is likely due to concentration of HCVpp being too low for surface bound particles to be detected by mAb staining and flow cytometry.

HCVpp are a useful tool for study of the role of HCV glycoproteins in virion attachment and entry in to cells. The ability of HCVpp to infect Hep3B cells (Fig. 3.31) indicates that these cells express all the molecules that are necessary for HCV entry. In contrast, none of the PBMC subsets studied here was infected by HCVpp, which could have indicated that these cells do not express all the molecules required for HCVpp attachment and entry. Alternatively, this may well have reflected an inability of the MLV-based pseudoparticles to transduce human PBMCs. It has been reported that nuclear import of DNA by MLV is dependent on the dissolution of the nuclear envelope during mitosis (Roe *et al.*, 1993), suggesting that MLV-based particles may be unable to transduce non-dividing cells such as naïve T and B cells, monocytes and DCs. Dardalhon *et al.* reported that up to 75 % of purified T cells could be transduced using a MLV vector after activation of the T cells (Dardalhon *et al.*, 2000). However, the

activation of PBMC subsets and monocyte-derived DCs was not found to be sufficient to enable the transduction of these cells by HCVpp. Had time permitted, further experiments would have been carried out with particles pseudotyped with e.g. the VSV-G protein, to check whether the inability of HCVpp to transduce PBMCs reflected an inability of MLV-based retroviral particles to express encoded genes within PBMC subsets, or failure of the HCVpp to bind to/enter PBMCs.

I found in this chapter that the pattern of binding of soluble truncated E2 proteins from all HCV strains tested (H77c, 1A14, BK and 1B12) to PBMCs was correlated with the level of cell surface CD81 expression. In line with the observed E2 binding pattern to different PBMC subsets, the binding of H77c $E2_{660}$ to PBMCs was substantially inhibited by blocking E2-CD81 interaction, suggesting that CD81 may play a prominent role in soluble E2 binding to PBMC subsets. How well soluble $E2_{660/661}$ proteins reflect the behaviour of native E2 is unclear; I thus also addressed the binding of HCV virions to PBMC subsets. Consistent with the results obtained for $E2_{660/661}$ binding to PBMCs, HCV virions also bound to PBMC subsets expressing high levels of CD81. The low levels of virion binding observed to PBMCs could have been due to the concentration of virions being too low for higher binding to be observed. Alternatively, the virions I used in this study might have a low CD81 binding ability as seen with 1A14 $E2_{661}$. I did not have the opportunity to carry out blocking studies to investigate the role of specific cell surface receptors in virion binding to PBMCs; nonetheless, these data provide suggestive evidence that CD81 may play an important role in the interaction of native E2 with PBMCs.

Results from blocking studies indicated that the binding of H77c $E2_{660}$ to some PBMC subsets (NK, NT and T cells) was almost entirely CD81-dependent; but E2 binding to other subsets (monocytes, DCs and B cells) was found to have an important CD81-independent component, indicative of E2 binding to additional receptor(s). Notably, those PBMC subsets which are reported to be infected by HCV *in vivo* were not those which expressed the highest levels of CD81, but were those found to bind H77c $E2_{660}$ via both CD81-dependent and CD81-independent mechanisms (Table 3.1). Thus although CD81 plays a predominant role in HCV binding to PBMC subsets, this interaction does not appear to be sufficient to allow infection of haematopoietic cells. This observation is in agreement with other studies suggesting that CD81 is required but not sufficient for the HCV attachment/entry process (Bartosch *et al.*, 2003a; Bartosch *et al.*, 2003b; Cormier *et al.*, 2004; Hsu *et al.*, 2003; McKeating *et al.*, 2004).

SR-BI is another E2-binding molecule that is thought to be involved in HCV infection of hepatic cells (Bartosch *et al.*, 2003b). Whilst CD81 is proposed to have a critical role as an attachment receptor, SR-BI is a strong candidate to mediate HCV internalisation into cells. SR-BI is known to internalise HDL and LPS to endosomal or Golgi compartments (Bocharov *et al.*, 2004a; Silver & Tall, 2001; Vishnyakova *et al.*, 2003); it may thus also mediate trafficking of bound virions into endosomes, wherein the pH dependent fusion event required for HCV infection of cells (Bartosch *et al.*, 2003b; Hsu *et al.*, 2003) could occur. I observed SR-BI expression on the majority of the PBMC subsets that are reported to be infected by HCV *in vivo* (Table 3.1). Further, I demonstrated using blocking mAbs that SR-BI makes an important contribution to H77c $E2_{660}$ binding to monocytes (Fig. 3.20). It is assumed that SR-BI also contributes to the binding of H77c $E2_{660}$ to other SR-BI^+ cell subsets such as plasmacytoid DCs, myeloid DCs and monocyte-derived DCs.

Table 3.1. Summary of CD81 and SR-BI expression on PBMC subsets, E2 binding to each subset and data from the literature addressing which PBMC subsets are infected with HCV *in vivo*.

Cell subsets	CD81 expression[1]	SR-BI expression[2]	E2 binding[3]	CD81-independent component of E2 binding[4]	Evidence of HCV infection in vivo
CD56dim NK	+++	NO	+++	NO	NO[5]
CD56high NK	+++	NO	++	NO	NO[5]
NT	++++	NO	++++	NO	?
Myeloid DCs	+	YES	++	YES	YES[6]
Plasmacytoid DCs	(+)	YES	+	YES	YES[6]
Monocytes	+	YES	++	YES	YES[7]
Naïve B	+	NO	+++	YES	YES[8]
Activated B	++	NO	+++	YES	YES[8]
CD4+ CD45RA+ T	+++	NO	++	NO	NO[5]
CD4+ CD45RO+ T	++++	NO	+++	NO	NO[5]
CD8+ CD45RA+ T	+++	NO	++++	NO	NO[5]
CD8+ CD45RO+ T	++++	NO	++++	NO	NO[5]
Monocyte-derived DCs	YES	YES	YES	YES	YES[9]

[1]Relative level of surface expression of CD81 as detected by staining with mAb JS-81 in the experiment shown in Figure 1. ++++ indicates a MFI of CD81-specific staining of >50; +++ a MFI of >30, ++ a MFI of >25, + a MFI of >15, and (+) a MFI of <10.

[2]YES indicates that SR-BI expression was detected on the indicated cell subsets; NO indicates that it was not.

[3]Relative level of E2 binding observed to PBMC subsets. Data from the experiment using H77c E2 in Figure 2. ++++ indicates a MFI of E2-specific staining >120; +++ a MFI of >80, ++ a MFI of >30, and + a MFI of <30.

[4]YES indicates that E2 binding to the PBMC subset concerned was not completely blocked by inhibition of E2-CD81 interaction; NO indicates that it could be.

[5]Lerat *et al.* (Lerat et al., 1998) and Muller *et al.* (Muller et al., 1993) detected no HCV RNA in NK/T cells, but did not subdivide $CD56^{dim}$ and $CD56^{high}$ NK cells, or $CD45RA^{+}$ and $CD45RO^{+}$ T cells.

[6]Goutagny *et al.* (Goutagny et al., 2003) detected HCV RNA in blood DCs, but did not subdivide myeloid and plasmacytoid DCs.

[7]Lerat *et al.* (Lerat et al., 1998) and Ducoulombier *et al.* (Ducoulombier et al., 2004) detected HCV RNA in monocytes.

[8]Lerat *et al.* (Lerat et al., 1998), Muller *et al.* (Muller et al., 1993), Goutagny *et al.* (Goutagny et al., 2003) and Ducoulombier *et al.* (Ducoulombier et al., 2004) reported HCV RNA in B cells, but did not subdivide naïve and activated B cells.

[9]Navas *et al.* (Navas et al., 2002) found HCV + and - RNA after incubating monocyte-derived DCs from healthy donor with HCV-positive serum.

Although SR-BI expression was detected on the majority of the PBMC subsets that are reported to be infected by HCV *in vivo*, B cells constituted a notable exception to this. I cannot exclude that B cells may express low levels of SR-BI that were not detected by the anti-SR-BI mAbs used here; but it is likely that at least the majority of the CD81-independent E2 binding observed to these cells was mediated via receptor(s) other than SR-BI. If B cells do become infected with HCV by a SR-BI-independent process, there may be an alternative pathway for virion delivery into endosomes in these cells. One possibility is that B cells may become infected with HCV via internalisation of virions bound to virus-specific surface immunoglobulins, a mechanism by which other viruses have been suggested to infect virus-specific B cells and impair the host anti-viral antibody response (Planz *et al.*, 1996).

Other evidence indicating that SR-BI may not be absolutely required for HCV entry into cells comes from the observation that a HCV clone lacking HVR-1 (which is thought to play a key role in E2-SR-BI interaction (Bartosch *et al.*, 2003b; Scarselli *et al.*, 2002)) was infectious in a chimpanzee model (Forns *et al.*, 2000). Thus the inability to bind SR-BI can be overcome by interacting with other receptor(s). It is thus speculated that E2 protein from different strains may have differential usage of several receptors to infect the host cells.

Some residual E2 binding to monocytes was still observed in the presence of both anti-CD81 and anti-SR-BI mAbs, indicating the existence of other receptor(s) mediating the interaction. Likewise factors in addition to CD81 and SR-BI are thought to be necessary for HCV entry into hepatocytes (Barth *et al.*, 2005; Bartosch *et al.*, 2003b; Hsu *et al.*, 2003; McKeating *et al.*, 2004). Other-E2 binding proteins that may be involved in HCV interaction with PBMC subsets are DC-SIGN and GAGs (Basu *et al.*, 2004; Lozach *et al.*, 2004; Ludwig *et al.*, 2004; Pohlmann *et al.*, 2003; Takikawa *et al.*, 2000). DC-SIGN

is expressed on a small proportion of $CD14^+$ cells in peripheral blood, but not on myeloid and plasmacytoid DCs (Engering *et al.*, 2002; Turville *et al.*, 2001), although it is highly expressed on immature monocyte-derived DCs (Baribaud *et al.*, 2002; Geijtenbeek *et al.*, 2000c). DC-SIGN thus likely played a part in the binding of H77c $E2_{660}$ observed to monocyte-derived DCs, and may also have contributed to E2 binding to a minor fraction of cells in the $CD14^{high}$ monocyte population I studied, but is unlikely to account for the majority of non-CD81, non-SR-BI mediated E2 binding to PBMC subsets. By contrast, GAGs are much more ubiquitously expressed (Kjellen & Lindahl, 1991), and are likely to contribute to E2 binding to multiple PBMC subsets. GAGs mediate the initial steps in the interaction of many viruses, including flaviviruses such as dengue virus (Chen *et al.*, 1997b), with host cells, and have likewise been suggested to act as a HCV capture receptor (Basu *et al.*, 2004; Takikawa *et al.*, 2000). However, although DC-SIGN and GAGs may contribute to E2 binding to PBMCs, again, they alone cannot mediate the infection of cells by HCV (Basu *et al.*, 2004; Lozach *et al.*, 2004).

Although HCV does infect haematopoietic cells, the liver constitutes the major site of *in vivo* viral replication. There are multiple steps in the viral lifecycle at which infection of PBMCs could potentially be restricted, but the observation that retroviral particles pseudotyped with the HCV E1E2 efficiently transduce hepatocytes but not PBMCs (Bartosch *et al.*, 2003a; Cormier *et al.*, 2004; Zhang *et al.*, 2004) suggests that viral attachment/entry into PBMCs may be suboptimal. I showed here that at least some PBMC subsets are capable of binding E2 via CD81, SR-BI and additional receptor(s); but it is plausible that the level of expression of SR-BI on PBMCs may limit their susceptibility to infection, and/or that PBMCs lack expression of additional factor(s)

required for efficient infection of hepatocytes. One drawback to the studies in which the ability of HCV pseudotyped retroviral particles to infect PBMCs was assessed is that none of the studies subdivided the PBMC into subsets, hence low level of infection of cell types present in limited numbers may well have been missed. Another drawback is that HIV-based vectors cannot undergo nuclear entry in monocytes (Neil *et al.*, 2001). Thus in experiments where the HIV-based vectors were used (Bartosch *et al.*, 2003a; Cormier *et al.*, 2004; Zhang *et al.*, 2004), failure to detect transduction of monocytes by pseudoparticles expressing HCV E1E2 might not reflect failure of pseudoparticle attachment/entry into monocytes. Monocytes may be one of the PBMC subsets most readily infected by HCV; they were found to bind E2 via both CD81, SR-BI and other receptor(s) (and were the PBMC subset on which I detected the highest level of SR-BI expression); further these cells have been reported as a site of infection *in vivo*. An alternative method needs to be used to investigate the potential for HCV infection of monocytes. Recently, Kaimori *et al.* showed that VSV-based pseudoparticles expressing HCV E1E2 enter myeloid DCs (Kaimori *et al.*, 2004). Thus VSV may provide a useful platform for the generation of pseudoparticles that can be used to study HCV cell entry into multiple cell types, including non-dividing cells.

Interestingly, all E2 proteins tested bound much less well to all PBMC subsets than H77c $E2_{660}$. This is likely due to the other E2s all binding much less well than H77c $E2_{660}$, to CD81 as shown by EIA (Fig. 3.22. and 3.26). That different HCV E2 proteins exhibit differential CD81 binding abilities has been reported in several previous studies: E2 proteins from a number of genotype 1b strains were found to exhibit a considerably lower level of binding to CD81 than that of the genotype 1a strains tested (Roccasecca *et al.*, 2003; Scarselli *et al.*, 2002; Triyatni *et al.*, 2002a; Yagnik *et al.*, 2000). However,

these studies employed genotype 1a E2s with only limited sequence diversity, e.g. E2s from clones H77c and H, the genetic distance between which is only 0.7 % (McKeating *et al.*, 2004). Here, I found that $E2_{660/661}$ proteins from two genotype 1a clones, C3 and 1A14, showed a low level of binding to $CD81^{high}$ PBMC subsets. Although it is unclear whether HCV expressing C3 E2 protein is infectious or not, retroviral pseudoparticles expressing E1E2 from the 1A14 clone have been shown to infect Huh-7 cells, indicating the functionality of this E2 sequence (Lavillette *et al.*, 2005a). These results indicate that soluble E2 protein from a functional genotype 1a HCV sequence can have a low affinity of binding to CD81, and that high affinity binding to CD81 is not a common feature of all genotype 1a E2s. Studies with larger panels of diverse genotype 1a E2s are required to determine whether there is any overall difference in the affinity of binding of genotype 1a and 1b E2s to CD81, or whether the extremely high CD81 binding affinity of H/H77c genotype 1a E2s may be anomalous.

Several previous studies have suggested possible CD81 binding sites within the E2 sequence. Yagnik *et al.* proposed on the basis of both experimental data and sequence analysis and modelling that there are two CD81 binding sites including residues aa 474-494 (binding region 1, near HVR-2) and aa 522-551 (binding region 2). Supporting this model, mAbs against epitopes near HVR-2 (aa 480-493; anti-E2 mAb 6/41a, and aa 544-551; anti-E2 mAb 6/53) were found to block soluble $E2_{660}$ binding to CD81 (Flint *et al.*, 1999b), and a mAb against aa 524-535 (anti-E2 mAb 9/75) has been shown to inhibit the binding of soluble $E2_{660}$, a full length E1E2 complex, VLPs, and retroviral pseudoparticles expressing E1E2 (all derived from the H/H77 strain) to CD81 (Clayton *et al.*, 2002; Hsu *et al.*, 2003; Owsianka *et al.*, 2001). In addition, several studies have reported that mAbs recognising aa 412-423 (anti-E2 mAbs AP33 and 3/11), which is near HVR-1, can inhibit E2 binding to CD81 (Clayton *et al.*, 2002; Hsu *et al.*, 2003;

Owsianka *et al.*, 2001). Roccasecca *et al.* also suggested that both HVR-1 and HVR-2 are involved in mediating the E2 binding to CD81 (Roccasecca *et al.*, 2003). Further, E2 sequence analysis and modelling showed that HVR-2/binding region 1 and aa 613-618 are physically very close in E2 structure, suggesting that aa 613-618 may also be involved in CD81 binding (Yagnik *et al.*, 2000). In support of this, Roccasecca *et al.* showed that removal of aa 613-618 of E2 from the H strain abolishes its ability to bind CD81 (Roccasecca *et al.*, 2003). Altogether, there are thus 4 putative regions responsible for CD81 interaction: (1) aa 412-423; (2) putative CD81 binding region 1 (aa 474-494) including HVR-2 (aa 474-482); (3) putative CD81 binding region 2 (aa 522-551), and (4) aa 613-618.

The protein sequences of H77c, 1A14, 1B12 and BK $E2_{660/661}$ were aligned using a CLUSTAL W program to reveal aa differences that may determine their differential affinity of binding to CD81 (Fig. 3.33). The protein sequences of H77c and 1A14 $E2_{660/661}$ shared 84.4 % homology. H77c $E2_{660}$ shared 77.6 % and 78.3 % aa identity with 1B12 and BK $E2_{661}$ respectively. 1B12 and BK $E2_{661}$ shared 85 % aa identity. All four sequences contained 16 conserved cysteine residues and 11 common predicted N-linked glycosylation sites. Apart from a few conserved aa residues within HVR-1, the aa sequences of HVR-1 and HVR-2 of the four E2s were very variable. By contrast, the region from aa 412-423 was highly conserved in all four E2 sequences; and the protein sequences of the region from aa 613-618 were all identical among the four E2 proteins. Several aa residues in putative CD81 binding region 2 differed between the E2s. These included aa residues at three sites identified in a study by Kronenberger *et al.*, 10 genotype 1a E2 sequences and 17 genotype 1b sequences were compared as typically differing between genotype 1a and 1b sequences (aa 531, 537 and 546) (Kronenberger *et al.*, 2004). Notably, at two of these aa positions, the aa residue present in the 1A14

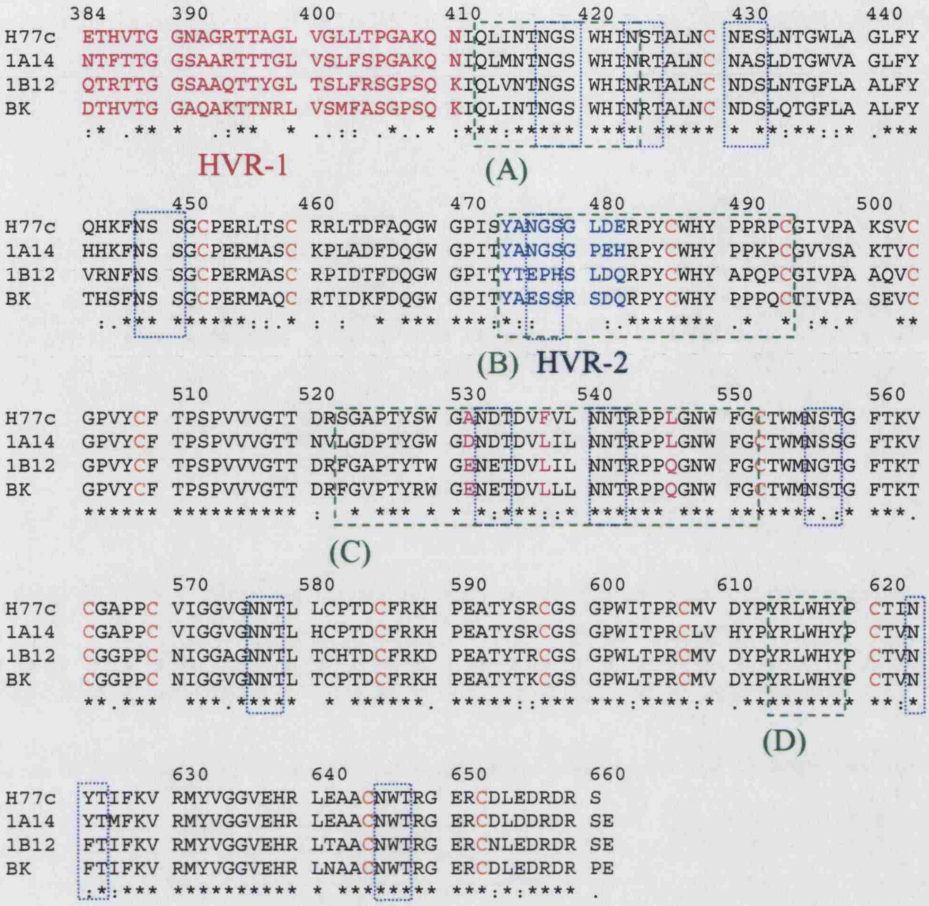


Figure 3.33. Alignment of the sequences of truncated $E2_{660/661}$ proteins from H77c, 1A14, 1B12 and BK HCV strains.

Alignment of the sequences of amino acid (aa) residues 384-660/661 of the soluble E2 proteins used in this study. H77c and 1A14 are genotype 1a and 1B12 and BK are genotype 1b strains. Hypervariable region (HVR)-1 (aa 384-410) and HVR-2 (aa 474-482) are indicated by red and blue lettering respectively. Cysteine residues are indicated in orange and the predicted N-linked glycosylation sites (NxT/S) by blue dotted boxes. * indicates an aa residue identical in all proteins, : indicates an aa residue with conserved substitutions, and . indicates an aa residue with semi-conserved substitutions. The green dotted boxes represent possible CD81 binding sites: (A) aa 412-423, (B) HVR-2 and CD81 binding region 1 (aa 474-494, Yagnik *et al.*, (2000)), (C) CD81 binding region 2 (aa 522-551, Yagnik *et al.*, (2000)), and (D) aa 613-618. The pink colour indicates residues thought to affect protein conformation (aa 531, 537 and 546, Kronenberger B *et al.*, (2004)).

sequence differed from that in the H77c sequence. At aa 531, H77c carried alanine (A), whilst 1A14, 1B12 and BK E2s carried acidic residues (aspartic acid (D) or glutamic acid (E)). However although glutamic acid at this position seems to be very conserved among genotype 1b clones, several genotype 1a clones, including HCV-1, which has a high CD81 binding affinity also carry a glutamic acid residue at this position (Choo *et al.*, 1991; Kronenberger *et al.*, 2004). At aa 537, H77c carried phenylalanine (F) and the rest of the E2 proteins (1A14, 1B12 and BK) carried leucine (L). According to Kronenberger *et al.*, leucine at aa 537 was very conserved among genotype 1b E2s (Kronenberger *et al.*, 2004). All reported sequences of genotype 1b E2 proteins with a low CD81 binding ability carried leucine at aa 537. Interestingly, all genotype 1a E2 sequences with a high CD81 binding ability carried phenylalanine at this position. Since a phenylalanine to leucine change could make a substantial structural difference, the low CD81 binding ability of 1A14 $E2_{661}$ could be partly due to the residue carried at aa 537. However, it is unlikely that just this one aa determines the difference in CD81 binding ability of the H77c and 1A14 E2s, as there are several regions of E2 involved in CD81 binding, and the conformation of the E2 binding regions could also be affected by aa changes outside these sites. Studies with larger panels of E2s from different genotypes are required to understand what determines the CD81 binding capacity (and infectivity) of HCV strains.

The interaction of the different E2 proteins with receptors other than CD81 was also briefly investigated in this chapter. H77c, C3, 1A14, BK and 1B12 $E2_{660/661}$ bound similarly to HepG2 cells, which express SR-BI but not CD81, indicating that all five $E2_{660/661}$ have the ability to bind receptors other than CD81. There was some indication that the 1B12 $E2_{661}$ may have a superior ability to bind to receptors expressed on

HepG2 cells as compared to the other $E2_{660/661}$ proteins (Fig. 3.27-B). However, due to problems in producing 1B12 $E2_{661}$, this result was obtained using only a single batch of protein. It would need to be confirmed with an independently-produced batch of 1B12 $E2_{661}$ before firm conclusions can be drawn from the data. Had time allowed, the binding of a range of doses of the different E2 proteins to HepG2 cells could have been analysed to give more insight into binding to SR-BI and other receptors on these cells.

Given the observation made in blocking studies that both CD81 and SR-BI contribute to H77c $E2_{660}$ binding to monocytes (Fig. 3.20), it might have been expected that H77c $E2_{660}$ would bind to $CD81^+$ $SR\text{-}BI^+$ hepatocyte cell lines better than other E2 clones. However, all $E2_{660/661}$ proteins tested (H77c, C3, 1A14, BK and 1B12) bound to Huh-7, Hep16 and Hep3B cells (which express both CD81 and SR-BI) at an equivalent level (Figs. 3.14, 3.23 and 3.27). This suggests that the expression of receptors other than CD81 on these cell lines may be high enough not to limit E2 binding. In support of this, retroviral particles pseudotyped with HCV E1E2 from clones whose E2 proteins have low and high CD81 binding abilities have been shown to infect $CD81^+$ $SR\text{-}BI^+$ hepatocyte cell lines well in *in vitro* assays (Bartosch *et al.*, 2003b; Lavillette *et al.*, 2005a; McKeating *et al.*, 2004; Zhang *et al.*, 2004).

Although the variation in CD81 binding affinity of E2 proteins from different viruses may not affect their infectivity, the interaction of E2 with CD81 and other cell surface proteins may be involved not only in viral entry but also in modulation of host cell responses. Notably, it has been reported that E2 cross-linking of CD81 on lymphocyte subsets *in vitro* can modulate their response to activating stimuli in a manner analogous to CD81 cross-linking via anti-CD81 mAbs, enhancing T cell activation in response to

signalling via CD3 (Wack *et al.*, 2001) and inhibiting NK cell activation (Crotta *et al.*, 2002; Tseng & Klimpel, 2002). On the basis of these observations, it is suggested that E2-CD81 interactions may modulate the host immune response *in vivo*, potentially contributing to viral persistence and/or pathogenesis. If this is the case, it might be predicted that viruses bearing E2 proteins that bind with differential affinity to CD81 may have correspondingly different immunomodulatory capacities, resulting in differences in persistence/pathogenesis. It is notable that overexpression of CD81 has been detected on B and T cells from HCV infected patients, which can be down-regulated during antiviral treatment (Kronenberger *et al.*, 2001; Zuckerman *et al.*, 2003), and this phenomenon is particularly pronounced in infections with genotype 1a viruses (Hofmann *et al.*, 2004; Kronenberger *et al.*, 2004; Zuckerman *et al.*, 2002). Although Zuckerman *et al.* reported that higher CD81 expression is associated with the development of HCV-related autoimmunity in chronic HCV patients (Zuckerman *et al.*, 2002), there have not been any studies that have documented differences in the pathogenesis of infections with HCV strains possessing E2s with differential CD81 binding ability, such as the severity of liver damage or spectrum of infection-associated autoimmune diseases. Thus the importance, if any, of the differential binding of HCV E2 proteins to lymphocyte subsets as a determinant of the *in vivo* disease course remains to be elucidated.

To gain further insight into how E2 interaction with CD81 on haematopoietic cells may potentially contribute to viral persistence/pathogenesis, in subsequent chapters I went on to address the effects of CD81 cross-linking on the activation and responses of NK cells and DCs.

Chapter 4 Effect of CD81 cross-linking on NK cell responses

4.1. Introduction

As shown in chapter 3, CD81 is expressed at high levels on lymphocyte subsets including T cells, NT cells and NK cells. On T cells, CD81 is known to mediate a co-stimulatory role, modulating T cell activation via the TCR (Wack *et al.*, 2001). However, at the time this project was started, the role(s) played by CD81 on NK cells were unknown. In this chapter, I set out to address this, with the longer-term goal of understanding the potential impact of interaction of the HCV E2 glycoprotein with CD81 on NK cells on the activation/functions of these cells.

NK cells were initially discovered through their ability to kill tumour cells without prior sensitisation of the host. They are now known to play an important role in provision of defence against many pathogens, particularly during the early phase of infection before the adaptive immune response becomes fully functional. NK cells kill their targets by release of granzymes and perforin (Henkart, 1994) or through interaction between surface proteins such as Fas-ligand and TNF-related apoptosis-inducing ligand (TRAIL) with death-domain-containing receptors on the target cell; in both cases, apoptosis is triggered in the target cell (Vujanovic *et al.*, 1996). NK cells also combat infections via production of antiviral cytokines, e.g. TNF-α and IFN-γ. In addition to their direct effector functions, NK cells also have an important immunoregulatory role, directing the nature of the adaptive response induced, together with DCs.

Classically, NK cells were found to kill target cells that expressed no or low levels of MHC class I molecules (Ljunggren & Karre, 1990). MHC class I molecules are normally ubiquitously expressed, but their expression is often down-regulated on tumour cells or virally-infected cells. Many viruses down-regulate surface MHC class I expression on the cells they infect to evade class I-restricted T cell killing (Tortorella *et*

al., 2000). This renders the cells more susceptible to NK killing. Thus the expression of MHC class I molecules serves as a positive indicator for the integrity of cells, protecting against NK cell attack (Bauer *et al.*, 1999). NK cells not only sense the lack of MHC class I molecules on target cells, but can also be triggered by interaction with ligands including non-classical MHC molecules, e.g. HLA-E and MHC class I chain-related molecules. Recognition of these ligands can lead to NK activation even in the presence of MHC class I expression (Cerwenka & Lanier, 2001).

A variety of NK cell receptors have been described; they include some that functionally inhibit NK cells and others that activate NK cells. NK cells are regulated by the balance of signalling through their activating and inhibitory NK receptors. In general, inhibitory receptors bind to particular classical MHC class I molecules, whereas activating NK receptors recognise non-MHC ligands or MHC class I-related molecules. All inhibitory receptors contain cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domains (Long, 1999). Upon the engagement of theses receptors, ITIMs recruit phosphatases that dephosphorylate crucial substrates of protein tyrosine kinases (PTKs) involved in the activation of NK cells (Lanier, 2003; Long, 1999). Such inhibitory receptors include KIRs and CD94/NKG2A in humans (Yokoyama & Scalzo, 2002).

In contrast, activating receptors have short cytoplasmic tails that lack ITIMs. Instead, these receptors contain charged residues in their transmembrane domains that enable them to interact with transmembrane adaptor molecules that carry immunoreceptor tyrosine-based activation motifs (ITAMs) or other signalling motifs. Numerous activating NK receptors have been identified, and they are summarised in Fig. 4.1. The

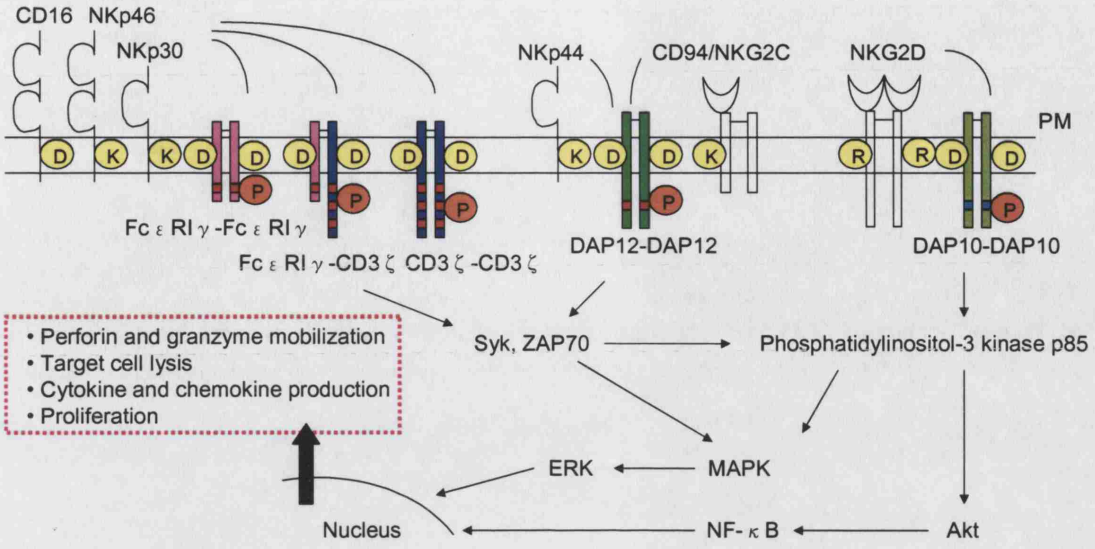


Figure 4.1. Pathways involved in signalling through activating NK cell receptors.

Activating NK cell receptors and their adaptor proteins are expressed on the plasma membrane (PM). Interaction between receptors and adaptor proteins is indicated by black lines, and the charged residues in their transmembrane domains that mediate the interaction are shown using the single-letter amino acid (aa) code. CD16, NKp46, and NKp30 can associate with FcεRIγ and CD3ζ. NKp44 and CD94/NKG2C can associate with DAP12. NKG2D can associate with DAP10. Transmembrane adaptor proteins FcεRIγ, CD3ζ and DAP12 bear 1 or 3 immunoreceptor tyrosine-based activating motifs (ITAMs; shown in red). FcεRIγ and CD3ζ can be expressed as homodimers or heterodimers whereas DAP12 is exclusively expressed as a homodimer. Ligation of ITAM-bearing receptor complexes leads to the recruitment and activation of the tyrosine kinases Syk and ZAP70. DAP10 is expressed as a homodimer and contains a YxxM motif (where x can be any aa, shown in blue) which binds to the p85 unit of phosphatidylinositol-3 kinase. The ligation of activating NK cell receptors induces the phosphorylation of signalling motifs and the activation of mitogen activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) or Akt and nuclear factor-κB (NF-κB). This in turn results in activation of NK cell effector functions, examples of which are shown (the combination of effector functions achieved depends on the signalling received). (Figure adapted from Sutherland *et al.*, (2002), Lanier (2003) and Vivier *et al.*, (2004))

ITAM-bearing adaptor molecules FcεRIγ, CD3ζ and DAP12 form either heterodimers or homodimers. Activating receptors such as CD16, NKp46, NLKp30, NKp44 and CD94/NKG2C associate with ITAM-bearing adaptor proteins that generate docking sites to activate the intracellular Syk family PTKs Syk and ZAP70. CD16 (FcγRIIIA) is an IgG receptor responsible for ADCC. NKp30, NKp44 and NKp46 are termed natural cytotoxicity receptors and their expression is strictly restricted to NK cells. NKp44 and NKp46 have been shown to bind the haemagglutinin proteins of influenza and certain other viruses; this interaction is mediated through sialic acid side-chains on NKp44 and NKp46 (Arnon *et al.*, 2001). CD94/NKG2C recognises a non-classical MHC class I molecule HLA-E (Braud *et al.*, 1998). NKG2D is unique among the activating receptors shown in Fig. 4.1, as it requires association with the adaptor protein DAP10 which does not carry an ITAM (Wu *et al.*, 1999). Instead, the cytoplasmic domain of DAP10 contains a Tyr-x-x-Met (YxxM; where x can be any amino acid) motif that binds to the p85 subunit of phosphatidylinositol-3 kinase (PI3 kinase) (Billadeau *et al.*, 2003; Wu *et al.*, 1999). NKG2D recognises MHC class I-related molecules such as MICA and MICB (Bauer *et al.*, 1999) and the UL16-binding proteins (ULBPs) (Sutherland *et al.*, 2002). MICA and MICB can be up-regulated on stressed, virus-infected and transformed cells. ULBPs were identified through their ability to bind to the human cytomegalovirus glycoprotein UL16 (Cosman *et al.*, 2001). ULBPs can be expressed by a wider range of cells, tissues and tumours than MICA/MICB (Cosman *et al.*, 2001).

Triggering of ITAM-based and/or PI3 kinase-based signalling pathways by activating receptors initiates NK cell cytotoxicity, cytokine gene expression and proliferation. It was believed that negative signalling generated by inhibitory receptors dominated over positive signalling from activating receptors. However, expression of ULBPs or MHC

class I-related molecules renders target cells susceptible to NK cell killing (Bauer *et al.*, 1999; Cosman *et al.*, 2001; Diefenbach *et al.*, 2001). This indicates that the positive signal delivered by NKG2D can override negative signals generated by engagement of inhibitory receptors. However, the fate of the target cell is controlled by the engagement of both inhibitory and activating receptors on the NK cell by their target cell ligands.

In addition to recognising ligands expressed on target cells, NK cells can respond to a number of different cytokines, including IFN-α/β, TNF-α, IL-2, IL-12, IL-15 and IL-18. These act on NK cells to cause up-regulation of cell-surface molecules involved in adhesion and migration, augumentation of cytotoxicity and/or the production of other cytokines such as IFN-γ. Some cytokines also regulate NK cell proliferation and survival/apoptosis. Various cytokines act at different stages of the immune response to expand NK cells and regulate their activity, thus controlling NK responses. Among the many cytokines that can stimulate NK cells, IL-2, IL-12 and IL-15 share similar, yet distinct, effects on NK functions, partly due to them sharing some common receptor chains (Azzoni *et al.*, 1998; Dunne *et al.*, 2001; Nguyen *et al.*, 2002). They activate NK cells through different STAT molecules. IL-18 is biologically similar to IL-1 but functionally similar to IL-12, inducing IFN-γ production from NK cells (Dinarello & Fantuzzi, 2003).

As reviewed in the main introduction, it has been shown that CD81 engagement can modulate T cell activation via the TCR and B cell activation through the BCR, indicating that CD81 modulates signalling through the classical antigen receptors expressed on these cells. Although activating NK cell receptors are not antigen specific, their receptor organisation appears to show an evolutionary conserved architecture

common to that of the TCR and BCR. I thus set out to address whether CD81 cross-linking could modulate the response of NK cells to stimuli delivered via activating receptors, in a manner analogous to its effects on T and B cell responses. During this project, Crotta *et al.* and Tseng *et al.* published results from studies addressing the role of CD81 on NK cells (Crotta *et al.*, 2002; Tseng & Klimpel, 2002). They found that cross-linking of CD81 on NK cells by an anti-CD81 mAb inhibited their activation in response to CD16 engagement or certain cytokines. In addition, they provided some evidence to suggest that CD81 cross-linking by HCV E2 could mimic the inhibitory effect of mAb cross-linking of CD81. I thus then focused much of my effort on investigating the effect of CD81 cross-linking on NK cell activation triggered through different activating NK cell receptors or cytokines.

Analysis of the role of CD81 on NK cells is of importance to increase understanding of one of the mechanisms by which NK cell functions are controlled; and also to give insight into the possible outcome of E2 interaction with CD81 on NK cells, a strategy that HCV may use to evade the antiviral activities of NK cells.

4.2. Results

4.2.1. Effect of CD81 cross-linking on the response of T cells to CD3 stimulation

Levy *et al.* reported that not all anti-CD81 mAbs deliver a co-stimulatory signal to lymphocytes when they bind to CD81, suggesting that the activating epitopes on CD81 are limited (Levy *et al.*, 1998). Thus before investigating the effect(s) of mAb cross-linking of CD81 on NK cells, the immunomodulatory activity of the commercially-available anti-CD81 mAbs (clones JS-81 and 1.3.3.22) was confirmed, and experimental conditions for cross-linking CD81 by means of Abs were optimised in

assays using T cells, following the method described by Wack *et al.* (Wack *et al.*, 2001). Purified T cells were stimulated in plates that had been coated with anti-CD3 mAb with or without one of the commercially available anti-CD81 mAbs (clone JS-81 or 1.3.3.22) or an isotype-matched (IgG1) control mAb, and the activation of $CD8^+$ and $CD4^+$T cells was assessed by analysis of up-regulation of the early activation marker CD69.

T cells were activated by the anti-CD3 mAb in a dose-dependent manner, as evidenced by up-regulation of CD69 expression on both $CD4^+$ and $CD8^+$ T cells (Fig. 4.2-A and-B). 50 % of T cells were stimulated to express CD69 at concentrations of anti-CD3 mAb between 0.3 and 1 μg/ml. No CD69 up-regulation was observed when suboptimal concentrations of anti-CD3 mAb (0.003 and 0.03 μg/ml) were used alone; however when used in combination with CD81 cross-linking, the sub-optimal concentrations of CD3 stimuli led to a substantial increase in the expression of CD69 on both $CD4^+$ and $CD8^+$ T cells (Fig. 4.2-A and-B). In the presence of anti-CD81 mAbs, 50 % of T cells were stimulated to express CD69 at concentrations of anti-CD3 mAbs between 0.003 and 0.03 μg/ml, approximately 2 logs lower than the concentration required to achieve this response when the anti-CD3 mAb was used alone. This effect was not seen when the anti-CD81 mAb was replaced with an isotype matched-control mAb (IgG1). Three-way interaction statistical analysis revealed that the difference observed in the T cell response to cross-linking by anti-CD3 and CD81 mAbs and by anti-CD3 and IgG1 was significantly different ($p<0.0001$).

Cytokines are important mediators of T cell effector functions; I thus also assessed the effect of CD81 cross-linking on IFN-γ production by T cells in response to anti-CD3 mAb. CD81 cross-linking enhanced IFN-γ production by T cells in response to suboptimal concentrations of anti-CD3 mAb (0.003 and 0.03 μg/ml) (Fig. 4.2-C).

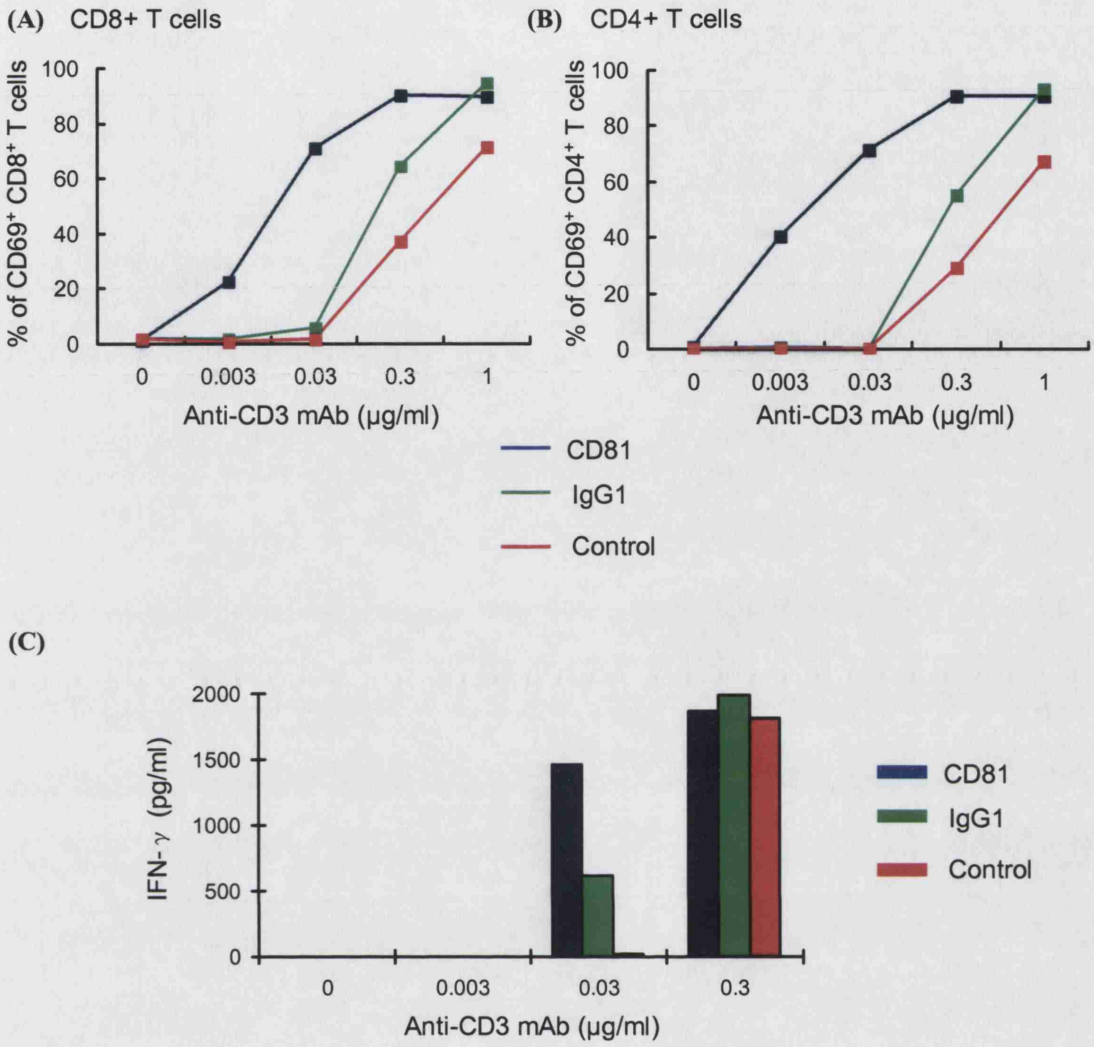


Figure 4.2. Effect of CD81 cross-linking on T cell activation in response to CD3 cross-linking.

Purified $CD3^+$ T cells were incubated for 24 hours (in experiments detecting CD69 expression) or 48 hours (in experiments detecting IFN-γ production) in plates that had been coated with the indicated concentrations of anti-CD3 mAb either alone (Control) or in combination with 5 µg/ml anti-CD81 mAb (clone JS-81; CD81) or an isotype matched control mAb (IgG1). The cells were then co-stained with anti-CD4, anti-CD8 and anti-CD69 mAbs, and CD69 expression on $CD8^+$ T cells (A) and $CD4^+$ T cells (B) was analysed by flow cytometry. Supernatants harvested from the cultures were analysed for IFN-γ content by ELISA (C). The results shown are representative of findings made in 4 independent experiments using PBMCs from different donors.

The results shown in Fig. 4.2 were obtained using anti-CD81 mAb clone JS-81, but a similar co-stimulatory effect on T cell responses to CD3 cross-linking was also seen using anti-CD81 mAb clone 1.3.3.22 (data not shown). As mentioned in Chapter 3, I found that anti-CD81 mAbs clone JS-81 and clone 1.3.3.22 compete with one another for binding to CD81 and thus likely recognise closely overlapping, if not the same, epitope(s) in the CD81 LEL. It was thus not surprising that both anti-CD81 mAbs were found to exhibit similar functional activity.

Overall, these results demonstrate that CD81 cross-linking by anti-CD81 mAbs (clones JS-81 and 1.3.3.22) specifically synergises with CD3-mediated T cell stimulation to lead to the up-regulation of an activation marker and IFN-γ production. These results are consistent with those reported by Wack *et al.* (Wack *et al.*, 2001).

4.2.2. Effect of CD81 cross-linking on the response of NK cells to CD16 stimulation

T cells and NK cells share very similar biological activities and there are a number of parallels in the signalling pathways involved in their activation. Given the role of CD81 in modulating signalling through the TCR on T cells, engagement of CD81 may also modulate the signals transduced via activating NK cell receptors. CD16 (FcγRIIIA), a low-affinity receptor for the Fc fragment of IgG, is expressed primarily on NK cells and is a major activating receptor on NK cells (Trinchieri & Valiante, 1993). Since CD16 shares some of the signalling components utilised by T cells in TCR signalling, I investigated the effect of CD81 cross-linking on CD16-mediated NK cell activation.

PBMCs or purified NK cells were stimulated in plates that had been coated with anti-CD16 mAb in combination with an anti-CD81 mAb (clone JS-81 or 1.3.3.22) or an

isotype-matched control mAb (IgG1), and the activation of NK cells was assessed by analysis of up-regulation of the early activation marker CD69 on $CD3^{-}CD56^{+}$ NK cells. I carried out 8 independent experiments using PBMCs from different donors, and in all experiments NK cells were activated by the anti-CD16 mAb in a dose-dependent manner (Fig. 4.3 and 4.4). In 3 of 8 experiments, cross-linking of cells by anti-CD81 mAbs (Fig. 4.3-A and -B) or control IgG1 (Fig. 4.3-C) did not alter the activation of NK cells induced by CD16. In 5 of 8 experiments, CD16-mediated up-regulation of CD69 on NK cells was partially inhibited by concentrations of anti-CD81 mAb of 1 μg/ml and above (Fig. 4.4-A). Statistical analysis revealed that the inhibitory effect exhibited by the anti-CD81 mAb cross-linking was significant ($p=0.045$). In experiments shown in Fig. 4.4 and most other experiments, the inhibitory effect of the anti-CD81 mAb was most apparent when PBMCs were stimulated with lower concentrations of anti-CD16 mAbs (0.3 and 1 μg/ml). However, very similar results were also obtained when the anti-CD81 mAb was replaced with an isotype-matched control mAb (Fig. 4.4-B). Statistical analysis with three-way interaction revealed that there was no significant difference in the NK cell response to cross-linking by anti-CD16 and CD81 mAbs and by anti-CD16 and IgG1 ($p=0.575$). In the experiment depicted in Fig. 4.4, there appeared to be some difference in the inhibitory activity of the anti-CD81 mAb and control IgG1 mAb at the lowest concentration used (1 μg/ml), however this was not statistically significant; further this was not reproducibly observed in all experiments carried out and may have been due to slight differences in the Ab concentration of the two preparations and/or the efficiency of Ab binding to the plates. Lower concentrations of anti-CD81 mAb or IgG1 (0.5 μg/ml and below) did not affect the activation of NK cells by anti-CD16 mAb (data not shown). The pattern of results shown was obtained regardless of the anti-CD81 mAbs used (clone JS-81 or 1.3.3.22), of the cell types

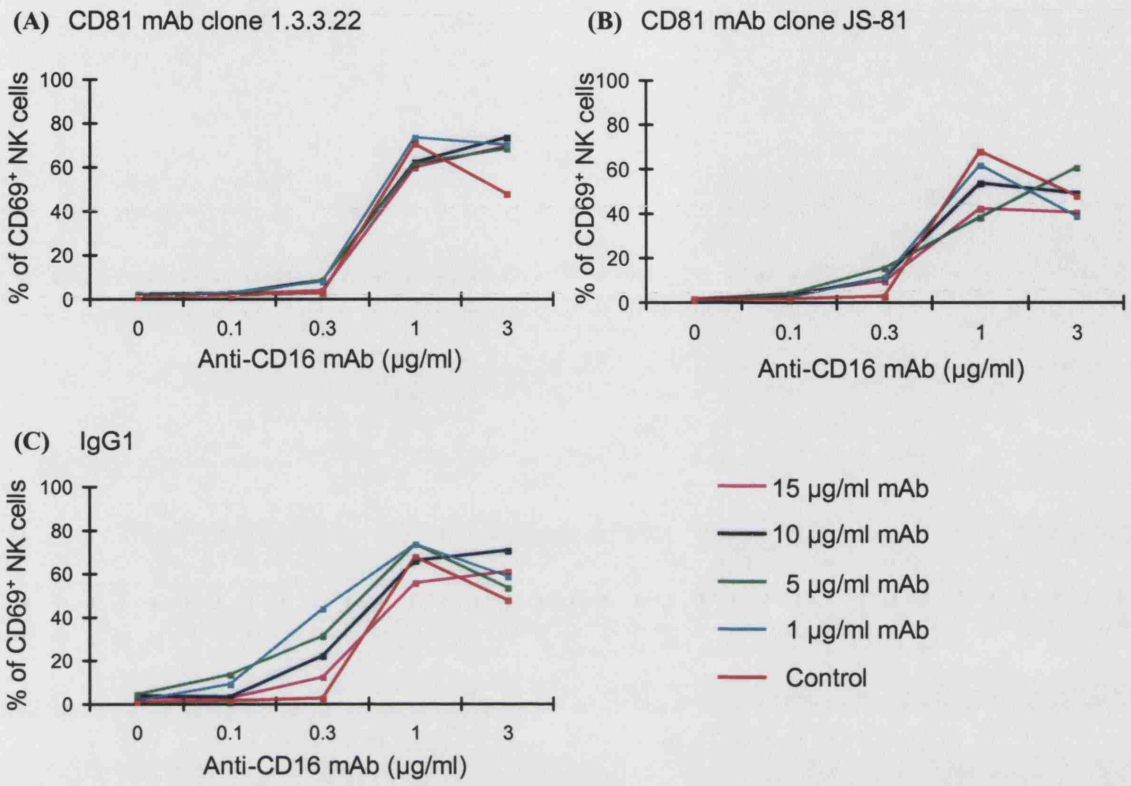


Figure 4.3. Analysis of the effect of CD81 cross-linking on NK cell activation in response to CD16 cross-linking.

PBMCs were incubated for 48 hours in plates that had been coated with the indicated concentrations of anti-CD16 mAb either alone (Control) or in combination with different concentrations (15 μg/ml, 10 μg/ml, 5 μg/ml or 1 μg/ml) of anti-CD81 mAb clone 1.3.3.22 (A), anti-CD81 mAb clone JS-81 (B) or an isotype control mAb (IgG1; (C)). The cells were then co-stained with anti-CD3, anti-CD56 and anti-CD69 mAbs, and CD69 expression on $CD3^{-}CD56^{+}$ NK cells was analysed by flow cytometry. The results are expressed as the % of NK cells that were $CD69^{+}$. The results shown are representative of findings made in 3 of 8 independent experiments carried out using PBMCs from different donors.

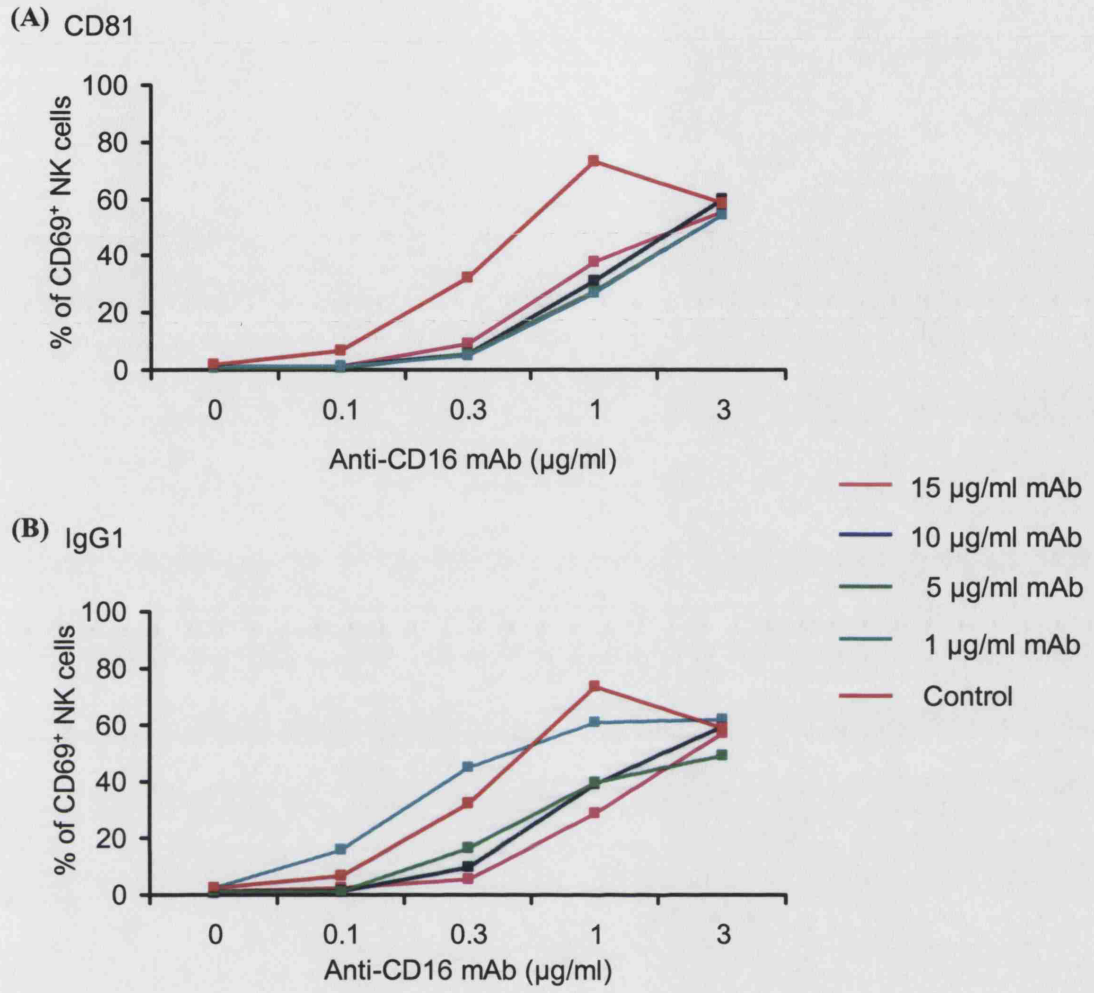


Figure 4.4. Analysis of the effect of CD81 cross-linking on NK cell activation in response to CD16 cross-linking.

PBMCs were incubated for 48 hours in plates that had been coated with the indicated concentrations of anti-CD16 mAb either alone (Control) or in combination with different concentrations (15 µg/ml, 10 µg/ml, 5 µg/ml or 1 µg/ml) of anti-CD81 mAb clone 1.3.3.22 (CD81, (A)) or an isotype control mAb (IgG1, (B)). The cells were then co-stained with anti-CD3, anti-CD56 and anti-CD69 mAbs, and CD69 expression on $CD3^{-}CD56^{+}$ NK cells was analysed by flow cytometry. The results are expressed as the % of NK cells that were $CD69^{+}$. The results shown are representative of findings made in 5 of 8 independent experiments carried out using PBMCs from different donors.

employed (NK cells within PBMCs or purified NK cells) or of the incubation periods (24 or 48 hours).

Overall, although some inhibition of CD16-mediated NK cell activation was observed in 5 of 8 experiments, this effect was mediated not only by the anti-CD81 mAb, but also by the isotype-matched control IgG1 mAb. Thus no specific effect of CD81 cross-linking on CD16-induced up-regulation of CD69 on NK cells was observed.

Since the inhibitory effect observed on CD69 expression on NK cells in response to CD16 cross-linking was not specific to anti-CD81 mAbs, I investigated whether CD81 engagement could block NK cell activity at a functional level. NK cells produce IFN-γ after exposure to many different stimuli. Thus I investigated whether cross-linking of CD81 could alter IFN-γ production by NK cells in response to CD16 cross-linking. Both PBMCs and purified NK cells produced IFN-γ in response to CD16 stimulation in a dose-dependent manner (Fig. 4.5). CD16 is expressed not only on NK cells but also on small populations of NT cells and T cells and on monocytes (Lanier, 1998), so in experiments using total PBMCs, some of the IFN-γ produced may have come from cell types other than NK cells; however in the experiment in Fig. 4.5-B, CD16-stimulated IFN-γ production by NK cells was directly addressed. CD81 cross-linking inhibited the IFN-γ production initiated by CD16 ligation not only from PBMCs (Fig. 4.5-A) but also from purified NK cells (Fig. 4.5-B). However, the isotype-matched control IgG1 mAb also inhibited the production of IFN-γ by PBMCs and purified NK cells in response to CD16. In the experiments using purified NK cells, anti-CD16 stimulated IFN-γ production was completely blocked by addition of either anti-CD81 mAb or IgG1. These results confirm that the inhibitory effect of anti-CD81 mAbs responses to CD16 stimulation is not specific to CD81 engagement. As discussed further at the end of the

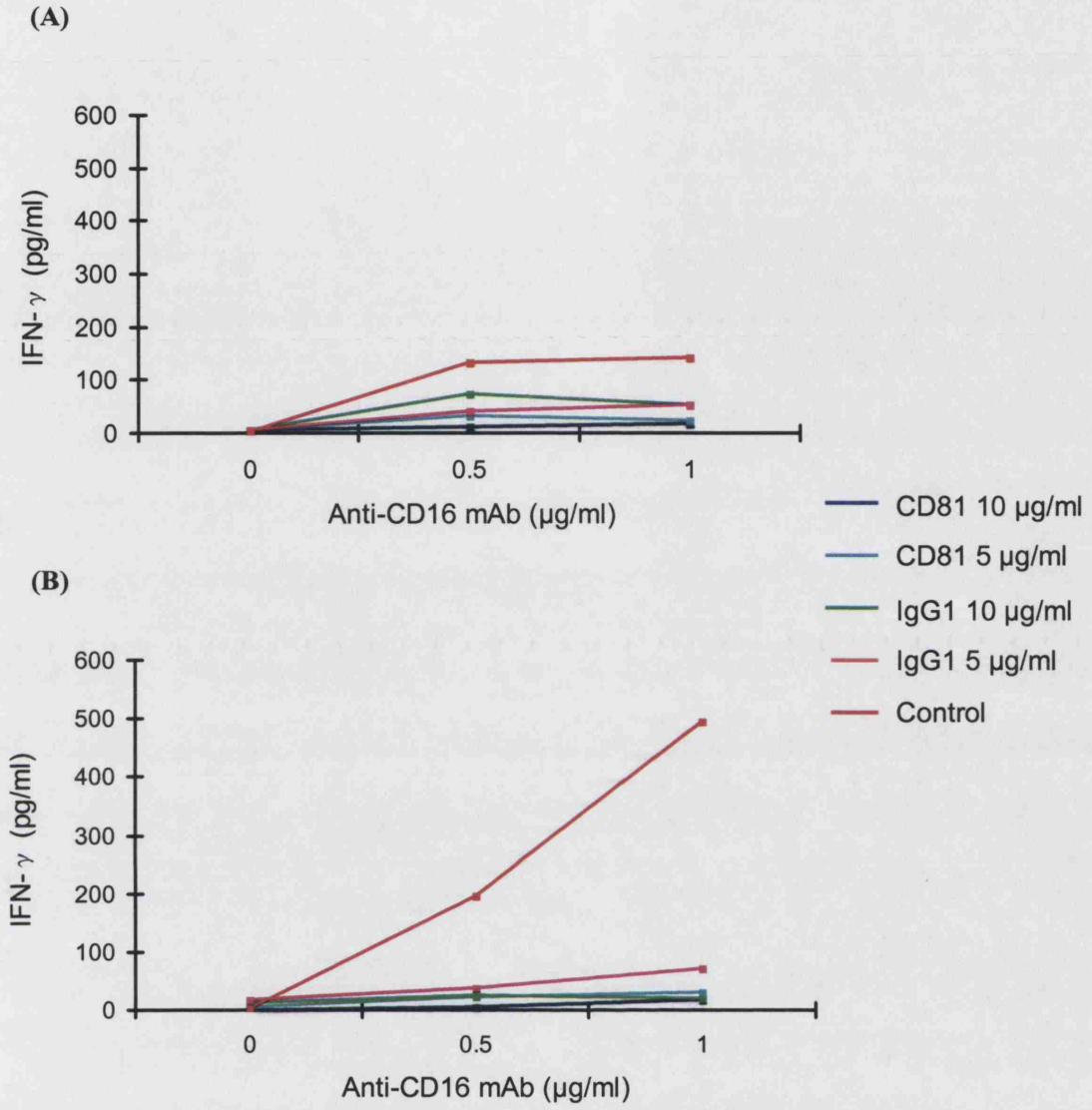


Figure 4.5. Analysis of the effect of CD81 cross-linking on IFN-γ production by NK cells in response to CD16 cross-linking.

PBMCs (A) or purified NK cells (B) were incubated in plates that had been coated with the indicated concentrations of anti-CD16 mAb either alone (Control) or in combination with 10 µg/ml or 5 µg/ml of anti-CD81 mAb clone JS-81 (CD81) or an isotype control mAb (IgG1). After 48 hours of incubation, supernatants were collected and analysed for IFN-γ content by ELISA. The results shown are representative of findings made in 2 independent experiments using PBMCs from different donors.

chapter, these results do not support the conclusions drawn by Crotta *et al.* and Tseng *et al.* who reported that CD81 cross-linking inhibits NK cell activation in response to CD16 stimulation.

4.2.3. Effect of CD81 cross-linking on the response of NK cells to NKG2C, NKG2D or CD161 cross-linking

There are numerous receptors on NK cells that transduce activatory signals using distinct adaptor proteins, as shown in Fig. 4.1. Having observed no specific effect of CD81 cross-linking on NK cell activation in response to CD16 stimulation, I chose to investigate whether CD81 cross-linking could modulate the NK cell response to stimuli mediated via the activating NK receptors CD94/NKG2C, NKG2D or CD161. NKD2C and NKG2D are associated with different adaptor proteins, activating NK cells through ITAM-dependent and -independent signalling pathways respectively (Billadeau *et al.*, 2003; Carretero *et al.*, 2000; Lanier *et al.*, 1998; Wu *et al.*, 1999). CD161 has been reported to mediate activatory or inhibitory signals upon cross-linking with mAbs (Jacobs *et al.*, 2005). The signalling pathway(s) via which it mediates its activity are not well defined, but it does not itself contain ITIMs/ITAMs, suggesting that it may associate with adaptor proteins.

Firstly, the cell surface distribution of NKG2C, NKG2D and CD161 on NK cells, NT cells and T cells was analysed. The majority of $CD56^{dim}$ and $CD56^{high}$ NK cells were found to express NKG2D and CD161, although there were also a small population of NK cells (predominantly $CD56^{dim}$ NK cells) that did not seem to express CD161 (Fig. 4.6-B). In contrast, the majority of NK cells did not express NKG2C, although there was a subset of both $CD56^{dim}$ and $CD56^{high}$ cells that did express this molecule (Fig. 4.6-B). The majority of NT cells were found to express NKG2D, whilst the majority of

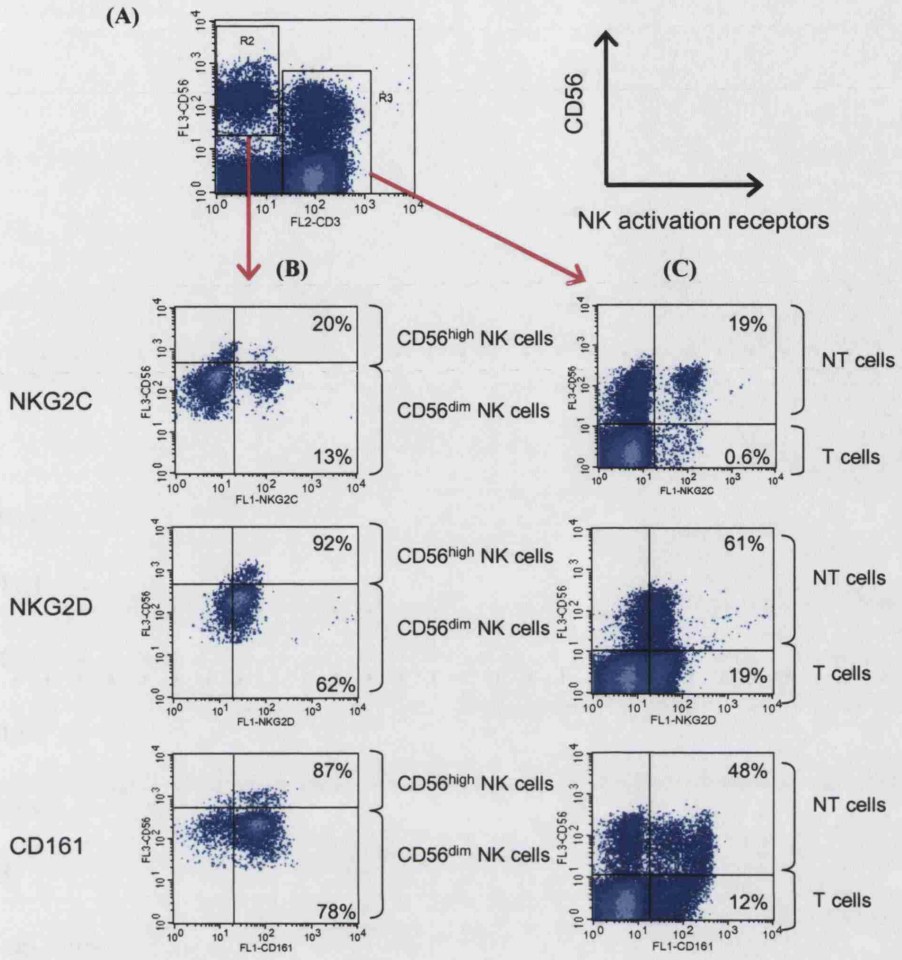


Figure 4.6. Expression of NKG2C, NKG2D and CD161 on NK cells, NT cells and T cells.

PBMCs were stained with mAbs to NKG2C (clone MAB1381), NKG2D (clone MAB139), or CD161 (clone DX12) followed by a secondary FITC-conjugated mAb. Cells were then stained with antibodies against CD3 and CD56 to allow identification of NK, NT and T cells. Dotplot (A) shows the CD3 versus CD56 staining profile of PBMCs and indicates the regions gated in the analysis below (R2: $CD56^{dim}$ and $CD56^{high}$ NK cells and R3: NT and T cells). The other dotplots show the expression of NKG2C, NKG2D and CD161 on $CD56^{dim}$ and $CD56^{high}$ NK subsets (B) or NT and T cell subsets (C). The % of cells in each subset which were positive for NKG2C, NKG2D or CD161 is shown. The position of the markers used to define positive staining was established based on the level of staining of cells with an isotype matched control mAb. The results shown are representative of 2 independent experiments using PBMCs from different donors.

NT cells did not express NKG2C, although there was a $NKG2C^+$ subset of NT cells (Fig. 4.6-C). Interestingly, three populations of NT cells expressing various levels of CD161 were observed: one expressing high levels of CD161, one expressing medium levels of CD161 and another expressing no CD161. There were small populations of T cells expressing NKG2D and CD161 (Fig. 4.6-C), but NKG2C was found to be expressed on very few T cells.

In initial experiments, I determined whether cross-linking of NKG2C, NKG2D or CD161 by mAbs, or in the case of NKG2D, a ligand, could activate NK cells. The NKG2D ligand employed was a MICA/Fc chimera, which consists of the extracellular domain of human MICA fused to the Fc region of human IgG. As controls, isotype-matched mAbs or IgG1/Fc, which consists of the Fc region of human IgG only, were used.

No up-regulation of CD69 expression on NK cells was observed when PBMCs were incubated for 24, 48 (data not shown) or 72 hours in plates that had been coated with mAbs to NKG2C, NKG2D or CD161, or the MICA/Fc chimera (Fig. 4.7). Incubation of PBMCs with these reagents also did not induce IFN-γ production (data not shown); further none of these treatments affected the cytolytic activity of NK cells against K562 cells (data not shown). In addition, stimulation of PBMCs with mAbs to NKG2C, NKG2D or CD161 in soluble form also did not activate NK cells (data not shown).

Andre *et al.* recently reported that activation of NK cells through NKG2D *in vitro* required addition of IL-2 to the culture supernatants (Andre *et al.*, 2004). I thus firstly determined a quantity of IL-2 that did not stimulate NK cells too strongly, but was sufficient to support NK cell activation through NKG2D signalling. It was found that incubation of PBMCs with 2 U/ml IL-2 induced the expression of CD69 on a low

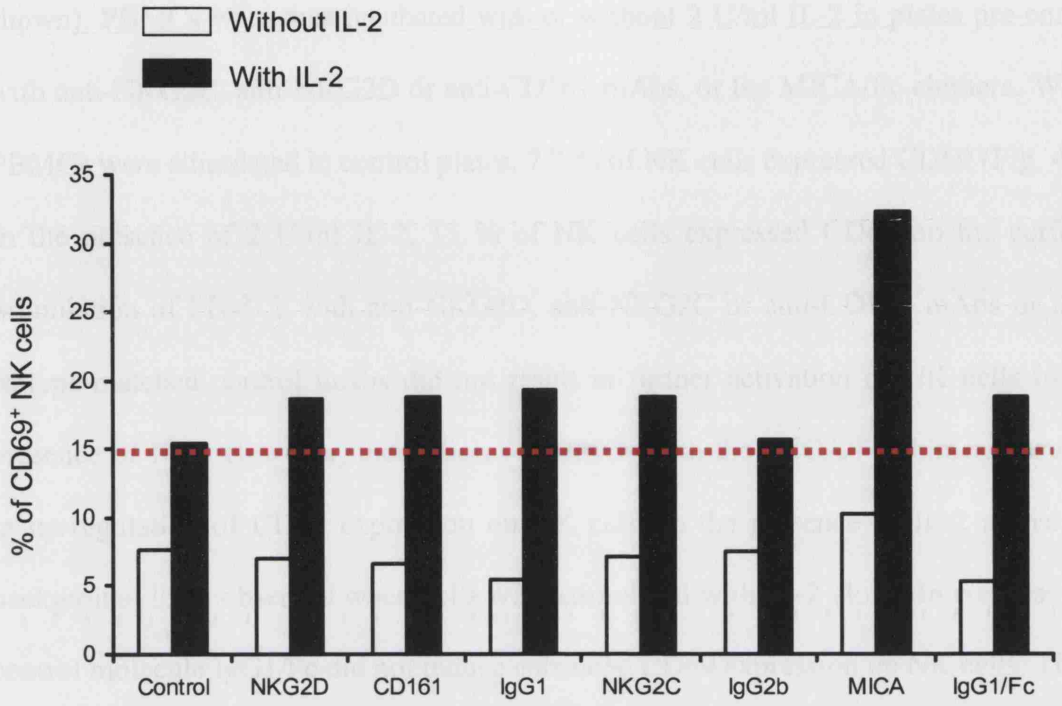


Figure 4.7. Analysis of NK cell activation in response to NKG2C, NKG2D or CD161 cross-linking.

PBMCs were incubated with or without 2 U/ml IL-2 in control plates (Control) or plates that had been coated with 10 μg/ml anti-NKG2D mAb (NKG2D), anti-CD161 mAb (CD161), anti-NKG2C mAb (NKG2C), MICA/Fc chimera (MICA), isotype-matched control mAbs (IgG1 or IgG2b) or isotype control molecule (IgG1/Fc). After a 72 hour incubation, the cells were stained with anti-CD3, anti-CD56 and anti-CD69 mAbs, and CD69 expression on $CD3^{-}CD56^{+}$ NK cells was analysed by flow cytometry. The results shown are the % of NK cells expressing CD69. The red line represents the background response to IL-2 stimulation. The data shown are representative of findings made in 2 independent experiments using PBMCs from different donors.

percentage of NK cells, but the majority of NK cells remained CD69 negative (data not shown). PBMCs were thus incubated with or without 2 U/ml IL-2 in plates pre-coated with anti-NKG2C, anti-NKG2D or anti-CD161 mAbs, or the MICA/Fc chimera. When PBMCs were stimulated in control plates, 7.7 % of NK cells expressed CD69 (Fig. 4.7). In the presence of 2 U/ml IL-2, 15 % of NK cells expressed CD69 on the surface. Stimulation of PBMCs with anti-NKG2D, anti-NKG2C or anti-CD161 mAbs or their isotype-matched control mAbs did not result in further activation of NK cells in the presence of IL-2. However, incubation of PBMCs with the MICA/Fc chimera resulted in up-regulation of CD69 expression on NK cells in the presence of IL-2 above the background level observed when cells were stimulated with IL-2 alone. In contrast, the control molecule IgG1/Fc did not induce enhanced CD69 expression on NK cells. These results indicated that cells could be activated through NKG2D cross-linking by the MICA/Fc chimera in the presence of IL-2. In addition, NK cells were activated by the MICA/Fc chimera in the presence of IL-2 in a dose-dependent manner (data not shown).

In further experiments, I investigated the effect of CD81 cross-linking on the activation of NK cells in response to NKG2D stimulation mediated by MICA/Fc chimera. PBMCs were incubated in plates that had been coated with MICA/Fc chimera with or without anti-CD81 mAb (clone JS-81) or its isotype control IgG1 either in the presence or absence of IL-2, and the level of CD69 up-regulation induced was assessed. In the representative experiment shown in Fig. 4.8, 32 % of NK cells expressed CD69 when PBMCs were stimulated with MICA/Fc chimera in the presence of IL-2. Incubation of cells in plates coated with IgG1 did not alter the activation of NK cells induced by MICA/Fc chimera ligation. Interestingly, when PBMCs were stimulated in plates that had been coated with MICA/Fc chimera and anti-CD81 mAb, the activation of NK cells

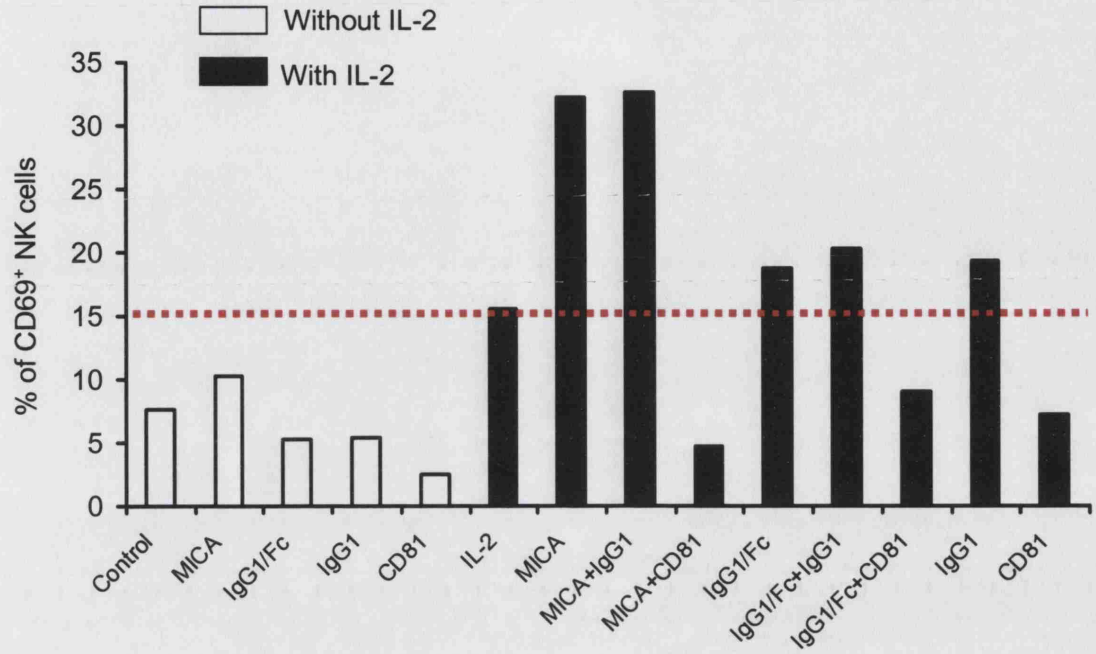


Figure 4.8. Effect of CD81 cross-linking on NK cell activation in response to MICA stimulation.

PBMCs were incubated with or without 2 U/ml IL-2 in control plates (Control) or plates that had been coated with 10 μg/ml MICA/Fc chimera (MICA) or isotype control molecule (IgG1/Fc) either alone or in combination with 10 μg/ml anti-CD81 mAb (clone JS-81; CD81) or an isotype-matched control mAb (IgG1). After a 72 hour incubation, the cells were stained with anti-CD3, anti-CD56 and anti-CD69 mAbs, and CD69 expression on $CD3^{-}CD56^{+}$ NK cells was analysed by flow cytometry. The results shown are the % of NK cells expressing CD69. The red line represents the background response to IL-2 stimulation. The data shown are representative of findings made in 2 independent experiments using PBMCs from different donors.

was inhibited down to the level of NK cell activation in control plates, which was less than the background response of cells to IL-2 alone. In this experiment, CD81 cross-linking also reduced the NK response to 2 U/ml IL-2 (either alone or in the presence of the control IgG1/Fc chimera) down to the control cell response level, an effect which was not observed with the isotype control IgG1 mAb. However, in other experiments, the isotype-matched control IgG1 mAb also inhibited the response of cells to IL-2 as much as the anti-CD81 mAb (data shown later in the chapter).

These results suggested that there may be a CD81-mediated inhibitory effect on NK cell activation in response to IL-2 stimulation and/or MICA/Fc ligation.

To provide further support for this, I attempted to investigate whether CD81 cross-linking altered the production of IFN-γ by NK cells in response to MICA/Fc ligation. However ligation of NKG2D by the MICA/Fc chimera was found not to enhance IFN-γ production by PBMCs in the presence of IL-2 (a low level of IFN-γ was produced by PBMCs in response to 2 U/ml IL-2 alone, but this was not increased following NKG2D ligation) (data not shown); thus it was not possible to address inhibition of the response to MICA/Fc ligation using this readout.

However, an effect of CD81 cross-linking on the NK cell response to MICA/Fc chimera ligation was also observed when CD56 expression on NK cells was analysed (Fig. 4.9). CD56, or neural cell adhesion molecule (N-CAM), is expressed on NK cells and NT cells, and is involved in adhesion and migration. Two subsets of NK cells ($CD56^{+}$ $CD3^{-}$ cells) can be distinguished on the basis of their level of CD56 expression; $CD56^{dim}$ NK cells, which typically comprise ~90 % of the total peripheral blood NK population, and $CD56^{high}$ NK cells. In the experiment shown in Fig. 4.9, 95.3 % of NK cells were $CD56^{dim}$ under control conditions, and 4.7 % were $CD56^{high}$ NK cells (Fig. 4.9-A).

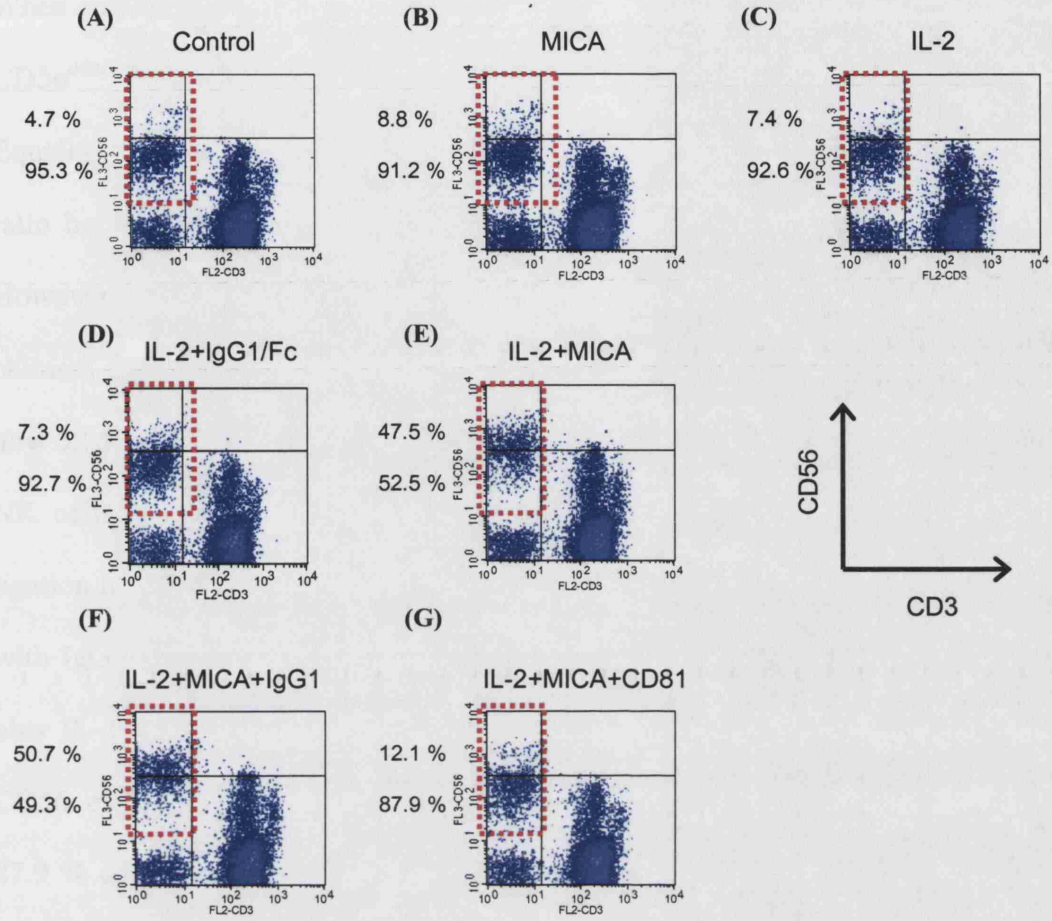


Figure 4.9. Effect of CD81 cross-linking on MICA+IL-2-induced up-regulation of CD56 expression on NK cells.

PBMCs were incubated with or without 2 U/ml IL-2 for 72 hours in control plates (Control) or plates that had been coated with 10 μg/ml MICA/Fc chimera (MICA) or isotype control molecule (IgG1/Fc) either alone or in combination with 10 μg/ml anti-CD81 mAb clone JS-81 (CD81) or an isotype-matched control mAb (IgG1). The cells were stained with anti-CD3 and anti-CD56 mAbs, and analysed by flow cytometry. The dotplots show the CD3 versus CD56 staining profile of cells treated as follows; Control (A), MICA alone (B), IL-2 alone (C), IL-2+IgG1/Fc (D), IL-2+MICA (E), IL-2+MICA+IgG1 (F), and IL-2+MICA+anti-CD81 (G). The quadrants allowing $CD56^{high}$ NK cells to be distinguished were set based on the level of CD56 staining of control NK cells (A). Cells within the regions indicated in red were gated to allow calculation of the % of $CD56^{dim/high}$ NK cells in each sample, which is indicated at the left side of each panel. The results shown are representative of findings made in 2 independent experiments using PBMCs from different donors.

When cells were stimulated with the MICA/Fc chimera or IL-2 alone, the ratio between $CD56^{dim}$ and $CD56^{high}$ NK cells underwent very little change (Fig. 4.9-B and -C). Equally, when cells were incubated with IL-2 in plates pre-coated with IgG1/Fc, the ratio between $CD56^{dim}$ and $CD56^{high}$ NK cells remained very similar to the control. However, incubation of cells with IL-2 in plates that had been coated with the MICA/Fc chimera resulted in up-regulation of CD56 expression on NK cells (Fig. 4.9-E), so that now 52.5 % of $CD56^{+}$ $CD3^{-}$ cells were $CD56^{dim}$ NK cells, while 47.5 % were $CD56^{high}$ NK cells. Up-regulation of CD56 expression on NK cells in response to MICA/Fc ligation in the presence of IL-2 was not altered when cells were exposed to plates coated with IgG1 (Fig. 4.9-F). However, when cells were stimulated with MICA/Fc chimera plus IL-2 in plates that had been coated with the anti-CD81 mAb, up-regulation of CD56 expression on NK cells was inhibited (Fig. 4.9-G). Under these conditions, 87.9 % of $CD56^{+}$ $CD3^{-}$ cells were $CD56^{dim}$ NK cells and only 12.1 % were $CD56^{high}$ NK cells. The observation that inhibition of CD56 up-regulation on NK cells in response to MICA/Fc chimera stimulation in the presence of IL-2 was mediated only by the anti-CD81 mAb, not by the isotype-matched control IgG1 mAb, suggested that the inhibitory effect was specific to CD81 ligation.

In conclusion, the results from these experiments suggested that CD81 cross-linking specifically inhibits the NK cell response (up-regulation of both CD69 and CD56 expression) to NKG2D cross-linking mediated by the MICA/Fc chimera in the presence of IL-2. However, I would ideally have liked to carry out further work to confirm this, including a more extensive analysis of NK cell activation in response to stimulation with a range of different concentrations of the MICA/Fc chimera in plates coated with

different quantities of anti-CD81 mAb or control IgG1 Ab; but unfortunately time did not permit this.

4.2.4. Effect of CD81 cross-linking on the response of NK cells to IL-2

In the majority of the experiments in section 4.2.3, CD81 cross-linking was not found to have any effect on the response of NK cells to IL-2 alone; however, it was possible that the inhibition of NK cell activation through NKG2D was due to modulation of signalling via IL-2, in addition to or instead of modulation of signalling via NKG2D. To gain more insight into whether the effects of CD81 ligation I had observed were specific to NK cell activation in response to NKG2D stimulation, I carried out further experiments to investigate whether CD81 cross-linking altered NK cell activation in response to stimulation with IL-2 alone.

PBMCs or purified NK cells were incubated in plates that had been coated with or without anti-CD81 mAb or IgG1, in the presence of different concentrations of IL-2. NK cells up-regulated CD69 expression in response to IL-2 in a dose-dependent manner (Fig. 4.10). CD81 cross-linking did not alter the activation of NK cells when PBMCs (Fig. 4.10-A) or purified NK cells (Fig. 4.10-B) were stimulated with IL-2. The isotype-matched control IgG1 mAb also did not alter CD69 up-regulation on NK cells in response to IL-2.

The effect of CD81 cross-linking on the NK cell response to IL-2 was also analysed by assessing IFN-γ production by NK cells, and up-regulation of perforin expression in these cells. Production of IFN-γ was increased in a dose-dependent manner when PBMCs (Fig. 4.11-A) or purified NK cells (Fig. 4.11-B) were activated by IL-2. CD81 cross-linking inhibited IFN-γ production by PBMCs and purified NK cells in response to IL-2 stimulation. However, ligation of cells with a control IgG1 mAb also inhibited

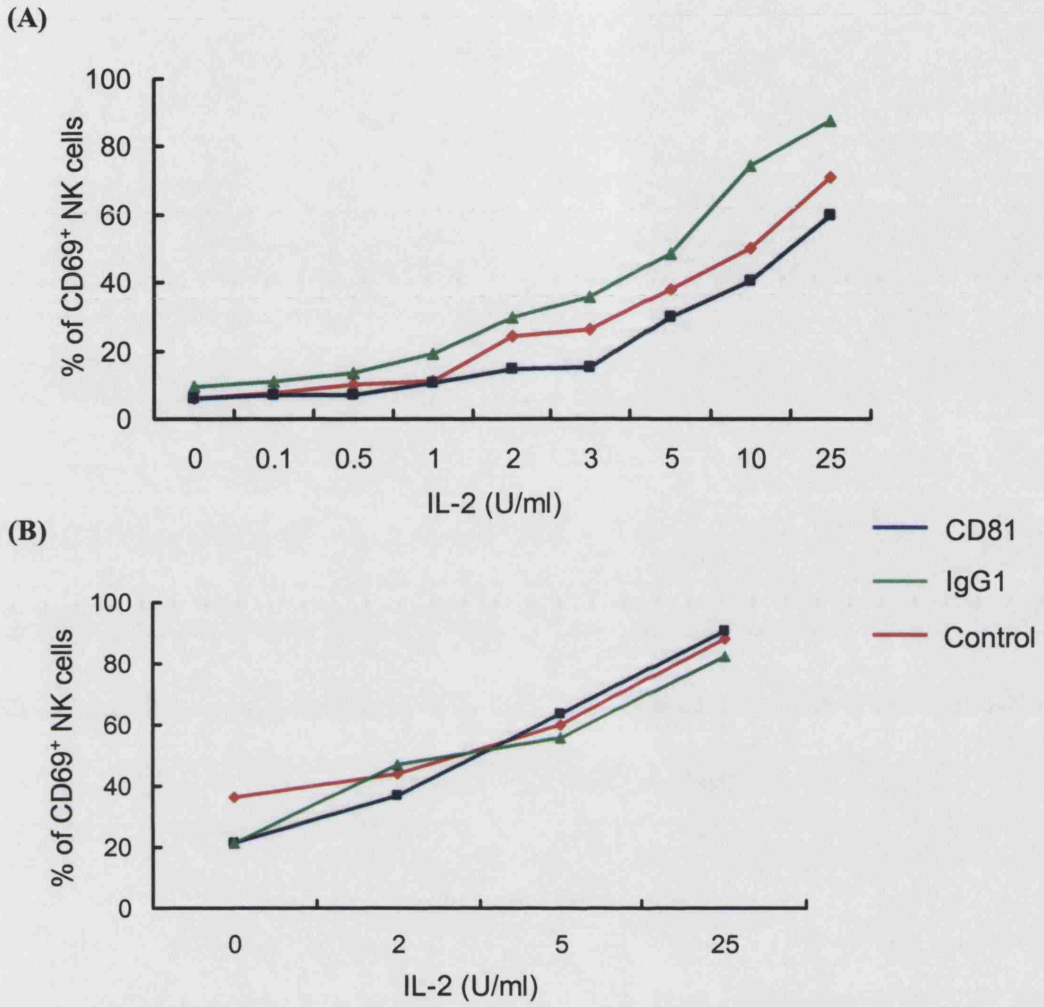


Figure 4.10. Effect of CD81 cross-linking on NK cell activation in response to IL-2 stimulation.

PBMCs (A) or purified NK cells (B) were incubated with the indicated concentrations of IL-2 in control plates (Control) or plates that had been coated with 10 μg/ml anti-CD81 mAb clone JS-81 (CD81) or an isotype-matched control mAb (IgG1). PBMCs were incubated for 72 hours and purified NK cells were incubated overnight. The cells were then co-stained with anti-CD3, anti-CD56 and anti-CD69 mAbs, and CD69 expression on $CD3^{-}CD56^{+}$ NK cells was analysed by flow cytometry. The results shown are the % of NK cells expressing CD69, and shown are representative of findings made in 3 independent experiments using PBMCs from different donors.

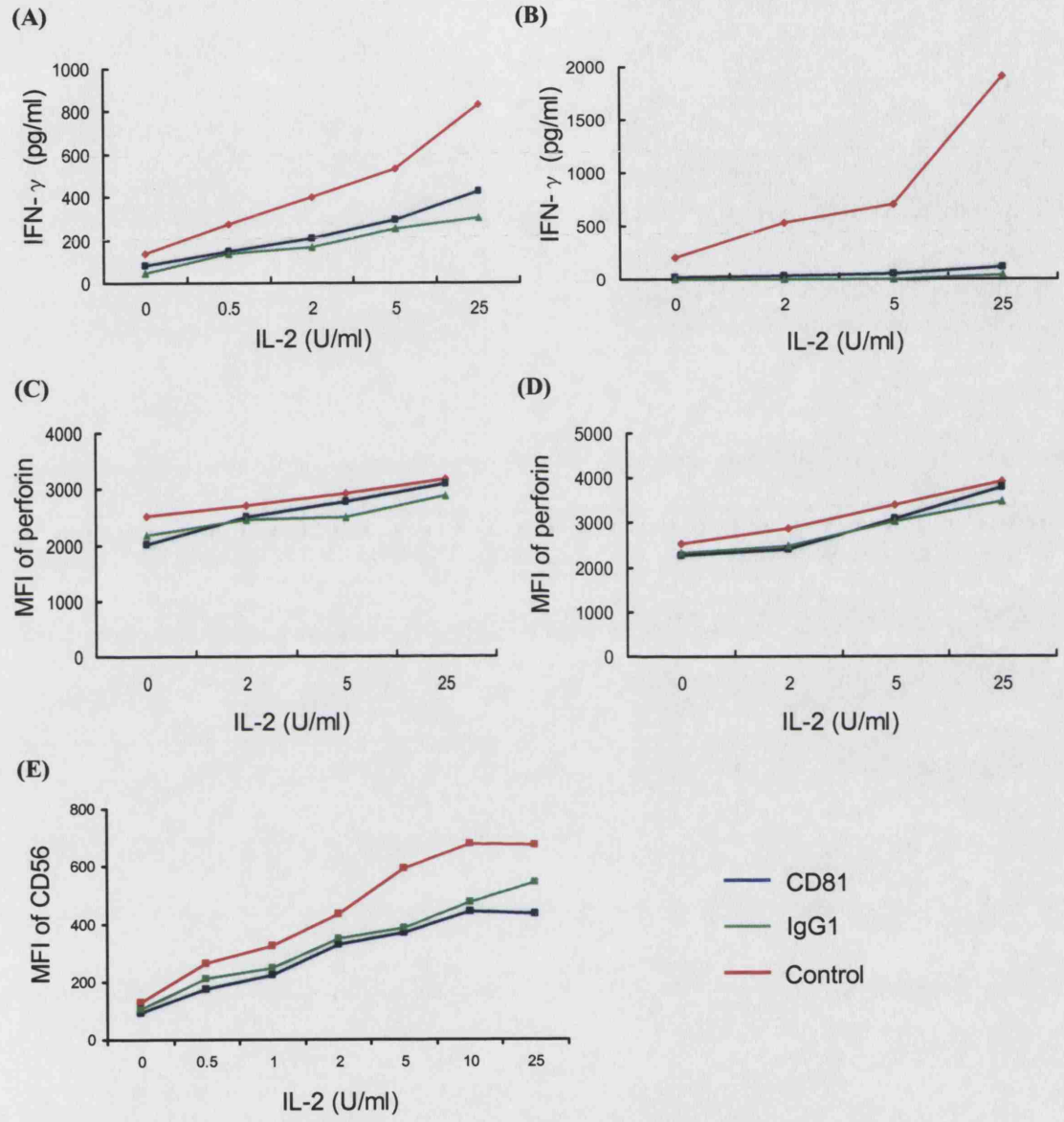

(A)
IFN- γ (pg/ml)
1000
800
600
400
200
0
0
0.5
2
5
25
IL-2 (U/ml)
(B)
IFN- γ (pg/ml)
2000
1500
1000
500
0
0
2
5
25
IL-2 (U/ml)
(C)
MFI of perforin
4000
3000
2000
1000
0
0
2
5
25
IL-2 (U/ml)
(D)
MFI of perforin
5000
4000
3000
2000
1000
0
0
2
5
25
IL-2 (U/ml)
(E)
MFI of CD56
800
600
400
200
0
0
0.5
1
2
5
10
25
IL-2 (U/ml)
CD81
IgG1
Control

Figure 4.11. Effect of CD81 cross-linking on IFN-γ production and perforin and CD56 expression by NK cells in response to IL-2 stimulation.

PBMCs (A, C and E) or purified NK cells (B and D) were incubated with the indicated concentrations of IL-2 in control plates (Control) or plates that had been coated with 10 μg/ml anti-CD81 mAb clone JS-81 (CD81) or an isotype-matched control mAb (IgG1). PBMCs were incubated for 72 hours (A) and purified NK cells were incubated overnight (B), then supernatants were harvested and analysed for IFN-γ content by ELISA. In (C) and (D), cells were stained with anti-CD3, anti-CD56 and anti-perforin mAbs after a 72 hour incubation period and perforin expression in $CD3^{-}CD56^{+}$ NK cells was analysed by flow cytometry. In (E), cells were stained with anti-CD3 and anti-CD56 mAbs after a 72 hour incubation period and CD56 expression on $CD3^{-}CD56^{+}$ NK cells was analysed by flow cytometry. In C, D and E, the results are expressed as the mean fluorescence intensity (MFI) of perforin or CD56 staining of NK cells. The results shown are representative of findings made in 2 independent experiments using PBMCs from different donors.

IFN-γ production by PBMCs and purified NK cells to a similar extent. Thus the inhibitory effect of the CD81 mAb on the production of IFN-γ by NK cells was not specific.

NK cells expressed a baseline level of perforin prior to stimulation. Exposure of PBMCs (Fig. 4.11-C) or purified NK cells (Fig. 4.11-D) to different concentrations of IL-2 increased the MFI of perforin-specific staining in NK cells in a dose-dependent manner. However, ligation of cells by either anti-CD81 mAb or control IgG1 had no effect on the up-regulation of expression of perforin in NK cells in response to IL-2.

Expression of CD56 was up-regulated when cells were activated by MICA/Fc chimera in the presence of a low concentration of IL-2, as shown in Fig. 4.10. Thus I investigated whether NK cells up-regulate CD56 expression in response to IL-2 alone, and whether CD81 cross-linking altered this response of NK cells. NK cells up-regulated CD56 expression in response to IL-2 in a dose-dependent manner (Fig. 4.11-E). Cross-linking of cells with anti-CD81 mAb or IgG1 resulted in the inhibition of CD56 up-regulation by NK cells in response to IL-2. Together, these results indicate that the there is no specific effect of CD81 cross-linking on NK cell activation in response to IL-2. These results are in contrast to findings made by Tseng *et al.* (Tseng & Klimpel, 2002).

4.2.5. Effect of CD81 cross-linking on the response of NK cells to IL-12, IL-15 and IL-18

NK cell activity is regulated by a number of other cytokines in addition to IL-2, for example IL-12, IL-18, IL-15 and IFN-α (Biron *et al.*, 1999). I thus investigated whether CD81 cross-linking had a specific effect on NK cell activation in response to cytokines other than IL-2.

PBMCs were stimulated in plates that had been coated with or without an anti-CD81 mAb or isotype-matched IgG1 control, in the presence of different concentrations of IL-12 (Fig. 4.12-A), IL-18 (Fig. 4.12-B) or IL-15 (Fig. 4.12-C). IL-12, IL-18 and IL-15 all stimulated up-regulation of CD69 expression on NK cells (Fig. 4.12). Cross-linking of CD81 by anti-CD81 mAb did not alter the up-regulation of CD69 on NK cells in response to IL-12, IL-18 or IL-15 and no change was observed when NK cells were exposed to immobilised IgG1 (Fig. 4.12). The MFI of CD69-specific staining on NK cells also showed no alteration after engagement of cells by anti-CD81 mAb or IgG1 (data not shown).

In summary, CD81 cross-linking did not alter the response of NK cells to IL-12, IL-18 and IL-15. Again, these results are in contrast to findings made by Tseng *et al.* (Tseng & Klimpel, 2002).

4.2.6. Effect of CD81 cross-linking on the response of NK cells to K562 cells

As described so far in this chapter, I focused mainly on investigation of the effects of CD81 cross-linking on NK cell activation induced by signalling through activating receptors or by cytokines. It is known that NK cells become activated and exhibit cytolytic activity on exposure to K562 cells. K562 cells are an erythroleukemia cell line derived from a chronic myeloid leukemia patient in blast crisis, which expresses very low levels of MHC class I molecules. At least one of the mechanisms by which NK cells are activated to kill K562 cells is thus via release of inhibitory signals.

I investigated whether CD81 cross-linking could modulate NK cell-mediated cytotoxicity against K562 cells using a standard chromium release assay.

An increasing % of specific target cell lysis, which was determined by the amount of ^{51}Cr released, was observed when K562 cells were incubated with increasing numbers

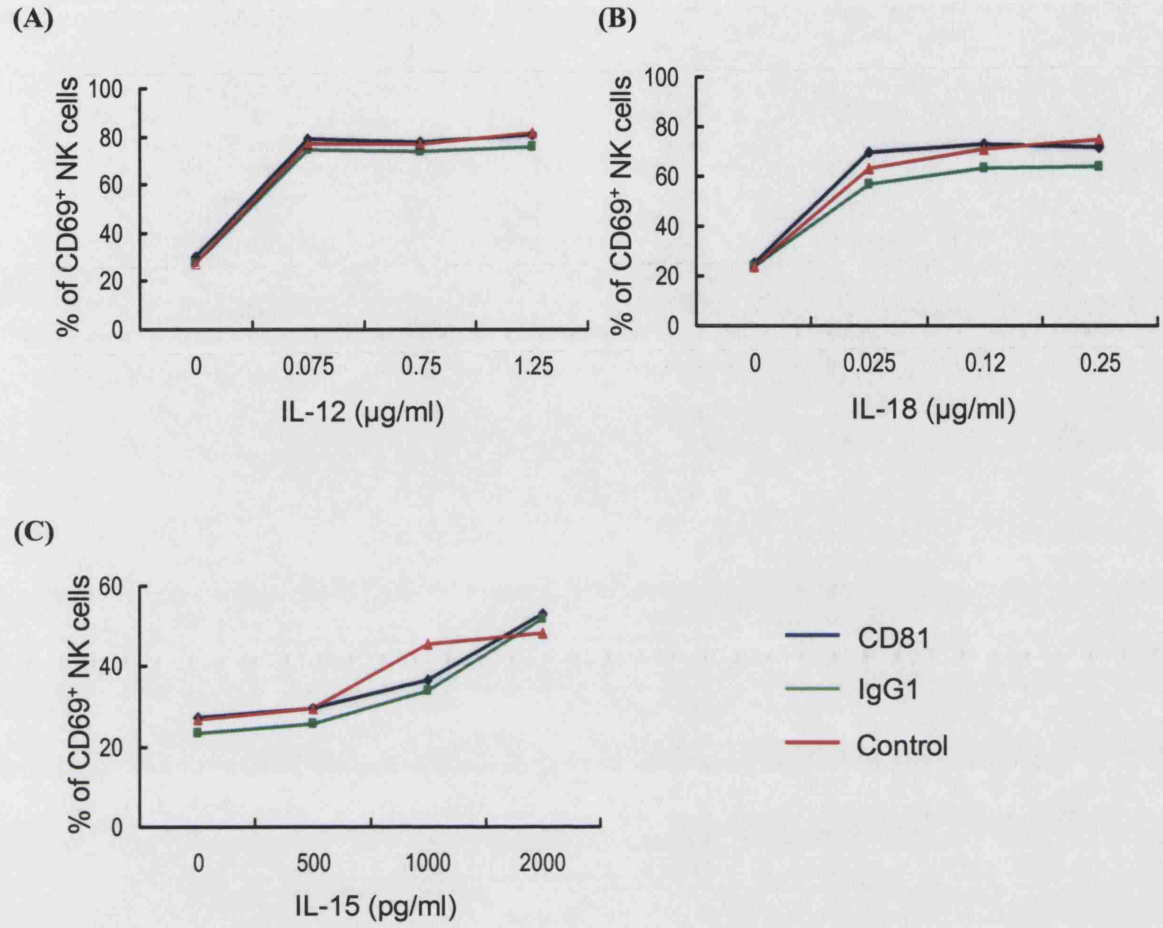


Figure 4.12. Analysis of the effect of CD81 cross-linking on NK cell activation in response to cytokine stimulation.

PBMCs were incubated overnight in control plates (Control) or plates that had been coated with 10 μg/ml anti-CD81 mAb clone JS-81 (CD81) or an isotype-matched control mAb (IgG1) in the presence of the indicated concentrations of the following cytokines: IL-12 (A), IL-18 (B) or IL-15 (C). The cells were then co-stained with anti-CD3, anti-CD56 and anti-CD69 mAbs, and CD69 expression on $CD3^{-}CD56^{+}$ NK cells was analysed by flow cytometry. The results are expressed as the % of NK cells that were $CD69^{+}$. The results shown are representative of findings made in 3 independent experiments using PBMCs from different donors.

of NK cells (Fig. 4.13). However, CD81 cross-linking did not affect the cytolytic activity of NK cells against K562 cells.

This indicates that there is no specific effect of CD81 engagement on NK cell activation in response to interaction with K562 cells. These results are in contrast to findings made by Tseng *et al.* (Tseng & Klimpel, 2002).

4.3. Discussion

CD81 is widely expressed on almost all cells. A hallmark of CD81 is its ability to associate with a wide range of membrane proteins, enabling CD81 to have different biological functions in different cell types. The biological functions of CD81 have been described on T cells (Imai & Yoshie, 1993; Mannion *et al.*, 1996; Wack *et al.*, 2001), B cells (Fearon & Carter, 1995), and more recently, on NK cells (Crotta *et al.*, 2002; Tseng & Klimpel, 2002). On T and B cells, CD81 modulates signalling through the classical antigen receptors, the TCR and BCR, respectively. The role(s) of CD81 on cells of the innate immune system had not been addressed when this project was started. In this chapter, I explored the role of CD81 on NK cells by investigating the effects of cross-linking of CD81 on NK cell responses to different stimuli.

CD81 is present in lipid rafts and associates with CD4, CD8, α4β1 integrins and CD82 on T cells (Imai & Yoshie, 1993; Mannion *et al.*, 1996). Although no physiological ligand for CD81 has been identified, the main function of CD81 has been suggested to be to co-ordinate the association of cell surface molecules and therefore facilitate the formation of efficient signalling complexes (Maecker *et al.*, 1997). Consistent with observations made in a previous study (Wack *et al.*, 2001), I found that cross-linking of CD81 on T cells by anti-CD81 mAbs (clones JS-81 or 1.3.3.22) enhanced $CD4^+$ and

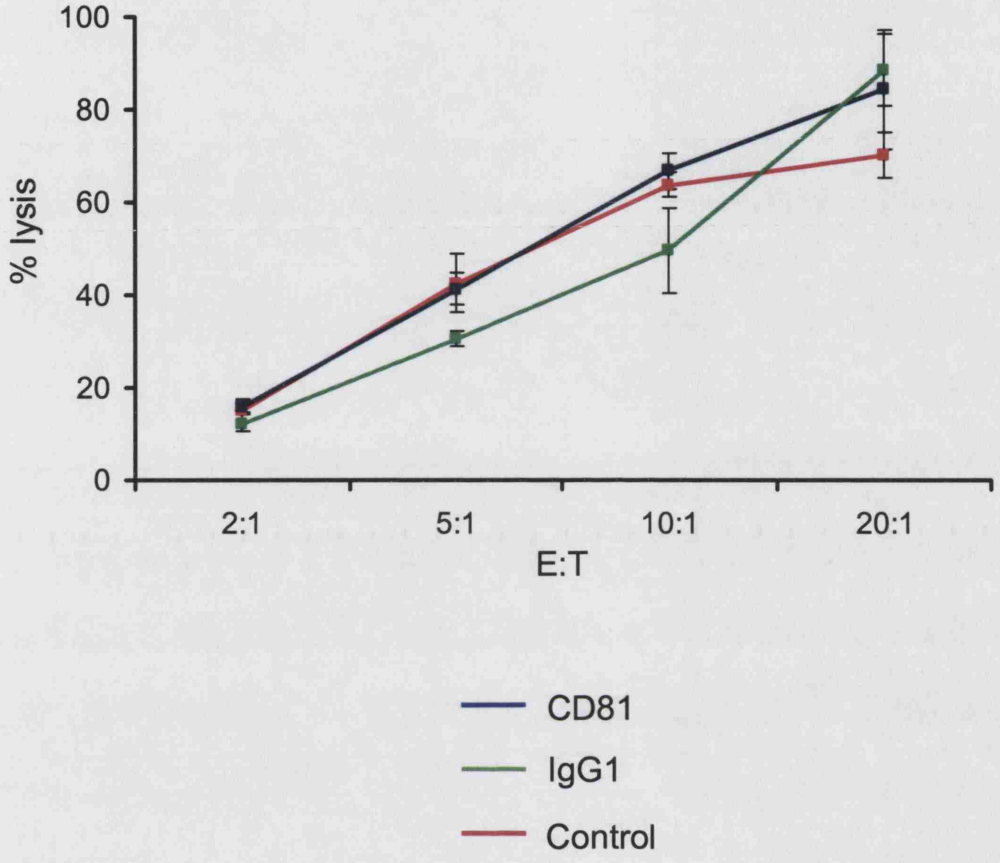


Figure 4.13. Analysis of the effect of CD81 cross-linking on activation of NK cell cytolytic activity following exposure to K562 cells.

Purified NK cells were incubated for 30 minutes in control plates (Control) or plates that had been coated with 10 μg/ml anti-CD81 mAb clone JS-81 (CD81) or an isotype control mAb (IgG1). ^{51}Cr-labelled target K-562 cells ($1.5x10^4$ cells per well) were then added to wells containing different NK cell numbers to give effector : target (E:T) cell ratios of 20, 10, 5 and 2:1. NK cell-mediated cytotoxicity was determined using a chromium release assay. The results are presented as the mean percentage of specific lysis, calculated as described in the Materials and Methods, and are representative of findings made in 3 independent experiments.

$CD8^+$ T cell activation in response to suboptimal concentrations of anti-CD3 mAb, as shown in Fig. 4.2. This effect was only mediated by the anti-CD81 mAbs and not by the isotype-matched control IgG1 mAb, strongly indicating that there was a CD81-specific effect on TCR-mediated signalling and subsequent T cell activation. CD81 cross-linking on T cells has been found to activate Lck and prolong anti-CD3-induced phosphorylation of CD3ζ, ZAP70 and LAT, indicating that the CD81 signal modulates the TCR signalling cascade at its most upstream steps (Soldaini *et al.*, 2003).

NK cells and T cells share similar phenotypic characteristics and biological functions. There are significant similarities in the signalling pathways activated by CD16 (FcγRIIIA) on NK cells and the TCR on T cells. Upon its cross-linking, CD16 is clustered and translocated into lipid rafts (Galandrini *et al.*, 2002) where it associates with phosphorylated ITAM-bearing adaptor proteins such as CD3ζ to send signals through ZAP70, as shown in Fig. 4.1. T cells and NK cells both utilise the CD3ζ chain as a coupling protein for their receptors. Considering this, it is logical to hypothesise that cross-linking of CD81 may modulate signalling induced by CD16 ligation on NK cells in a similar way to that in which it modulates signalling induced by TCR ligation on T cells.

In some experiments, ligation of CD81 on NK cells by anti-CD81 mAbs (clones JS-81 or 1.3.3.22) was found to reduce CD16-induced up-regulation of CD69 expression and IFN-γ production by NK cells; however, ligation of cells with an isotype-matched control mAb IgG1 also reduced CD16-induced NK cell activation to a comparable extent as was observed with the anti-CD81 mAbs. These results indicated that the inhibitory effect of the anti-CD81 mAbs on the NK cell response to CD16 cross-linking

was not specific. It may instead have been mediated via competition for Ab binding to the plates (with the CD81 and control IgG1 mAbs reducing the density of CD16 mAb binding to the plates to a level where CD16 cross-linking was suboptimal); or via an Fc-receptor-mediated effect (with the Fc portion of the CD81 and control IgG1 mAbs competing with the anti-CD16 mAb for binding to CD16, but not inducing sufficient CD16 cross-linking to deliver an activating stimulus, or possibly even delivering an inhibitory signal through the Fc receptor). In support of the latter hypothesis, several previous studies have documented the inhibition of NK cell activity by incubation of cells with monomeric human IgG (Gherman *et al.*, 1987; Sulica *et al.*, 1982; Sulica *et al.*, 1996; Sulica *et al.*, 1993). Sulica *et al.* reported that incubation of NK cells for 2-3 days with anti-CD16 mAb clone 3G8 (isotype IgG1) in the presence of IL-2 reduced their cytolytic activity against K562 cells (Sulica *et al.*, 1996). The inhibition of NK cell activity was found to be: (1) associated with increased levels of intracellular cyclic AMP (Bancu *et al.*, 1988) and (2) caused by the Fc region of the IgG molecules, since the binding site on CD16 is shared between anti-CD16 mAb clone 3G8 and the Fc portion of IgG (Gherman *et al.*, 1987; Sulica *et al.*, 1993). Galatiuc *et al.* also supported the idea that the Fc portion of IgG1 mAb might play a role in down-regulating NK activity by showing that $F(ab')_2$ fragments of the anti-CD16 mAb clone 3G8 were more effective than the intact mAb in augmenting NK cytolytic activity (Galatiuc *et al.*, 1995). Further Galatiuc *et al.* found that the outcome of CD16 cross-linking on NK cells by anti-CD16 mAb depends on the individual: NK cells from some donors showed augmented cytotoxic activity following exposure to an anti-CD16 mAb, whilst cells from other donors were inhibited by the same mAb (Galatiuc *et al.*, 1995).

The Abs used here were mouse IgG1 mAbs, not human Abs; however Song *et al.* reported that human NK cells can recognise mouse IgG molecules and that ADCC could be elicited by mouse IgG Abs (Song *et al.*, 1990). Together these observations suggest the possibility that the Fc portion of the mouse IgG1 mAbs used in this study (anti-CD81 mAb or control IgG1 mAb) might bind to CD16 on NK cells (possibly after dissociation from the plate), which may result in: (1) inhibition of NK cell activity or (2) reduction of NK activation by blocking the binding of anti-CD16 mAb. In addition to CD16, human NK cells express another type of Fc receptor, CD32 (FcγRIIc) (Metes *et al.*, 1999). CD32 can also act as a receptor for murine IgG1 (Galatiuc *et al.*, 1995), however, the consequence of ligation of CD32 on NK cells by mouse IgG1 has not been characterised. If time had allowed, I would have carried out experiments using $F(ab')_2$ fragments of the anti-CD81 mAb instead of intact mAb to cross-link CD81, to see whether this failed to mediate inhibition of NK cell activation in response to CD16 stimulation. Alternatively, an anti-CD81 mAb of a different isotype (not IgG1) could be used.

Crotta *et al.* and Tseng *et al.* reported that CD81 cross-linking on NK cells had an inhibitory effect on their response to CD16 stimulation (Crotta *et al.*, 2002; Tseng & Klimpel, 2002). By contrast, I saw no effect, or a small effect that was also observed with the isotype control mAbs. There are several differences between my studies and those reported by Crotta *et al.* and Tseng *et al.* both in the experimental design and reagents used. Firstly, Crotta *et al.* used cultured or cloned NK cells in the majority of their experiments. It is not clear whether the phenotypes and functions of cultured and cloned NK cells are the same as those of NK cells purified from fresh blood, which I used. Secondly, although Crotta *et al.* and Tseng *et al.* both used anti-CD81 mAb clone JS-81, which I also used in this study, they did not show the results obtained using an

irrelevant isotype-matched control mAb. Instead, for some but not all experiments, they showed the results obtained using an anti-CD56 mAb in place of the anti-CD81 mAb. Thirdly, Crotta *et al.* mentioned in the text of their paper that the inhibitory effect of CD81 cross-linking was detectable over a broad range of Ab concentrations (2.5-20 μg/ml), with 10 μg/ml giving the most potent and consistent inhibition compared with controls. However, they did not show the effect of isotype-matched control mAbs at different concentrations as I did in this study; further, they showed data obtained using one concentration of anti-CD81 mAb in most experiments. I could have selected one set of conditions where the anti-CD81 mAb and isotype-matched control mAb showed different effects (such as 1 μg/ml of anti-CD81 and control mAbs in Fig. 4.4). Thus it is not clear whether the observations reported by Crotta *et al.* and Tseng *et al.* are really indicative of a specific effect of CD81 cross-linking on the response of NK cells or not. Interestingly, although CD81 cross-linking rendered T cells responsive to anti-CD3 mAb concentrations ~2 logs lower than those at which activation (as measured by CD69 up-regulation) was observed under control conditions, the inhibitory effect of CD81 cross-linking on up-regulation of CD69 on NK cells was much more minor in my experiments. In line with this, Crotta *et al.* frequently observed only a partial inhibition of NK activation induced by CD16 stimulation. Thus if CD81 cross-linking does have an inhibitory effect on the NK cell response, it may be very minor, making it difficult to distinguish from the Fc-mediated inhibitory effect of control IgG1 Abs.

I also found that CD81 cross-linking on NK cells did not have any specific effect on cytokines such as IL-2, IL-12, IL-15 and IL-18, or to K562 cells. The production of IFN-γ by NK cells in response to IL-2 was reduced by incubation of purified NK cells with anti-CD81 mAb or isotype-matched control mAb, indicating that this effect might

also be mediated through Fc receptors. In addition, CD81 cross-linking on NK cells did not inhibit their cytolytic activity against K562 cells. These results are again in contrast to findings made by Tseng *et al.* They reported that CD81 cross-linking inhibited IL-2, IL-12 or IL-15-stimulated production of IFN-γ by NK cells and the cytolytic activity of NK cells against K562 cells (Tseng & Klimpel, 2002). Crotta *et al.* and Tseng *et al.* concluded that ligation of CD81 can inhibit NK cell activation in response to diverse stimuli, which trigger NK cell activation via different signalling pathways: signalling through ITAMs triggered by CD16 ligation; signalling initiated by the absence of engagement on NK inhibitory receptors which was seen in a killing assay using K562 cells; and signalling through STAT molecules triggered by cytokines. Whilst possible, this seems a somewhat surprising finding.

Crotta *et al.* and Tseng *et al.* also presented some data indicating that inhibition of NK cell responses could be achieved by exposure to immobilised soluble truncated HCV E2 proteins (Crotta *et al.*, 2002; Tseng & Klimpel, 2002). Notably however, the CD81-dependence of this effect was not demonstrated. Had time allowed, I would also have liked to investigate the effects of plate-bound HCV E2 proteins on NK cell responses. In these experiments, I would have used an anti-E2 mAb of an isotype other than IgG1 to capture and present the E2 protein on plates, so that the capture Ab did not interfere with binding of anti-CD16 mAbs to the NK cells. It would also have been of great interest to carry out experiments with soluble E2s exhibiting both high and low binding to CD81.

NKG2C, NKG2D and CD161 have all been identified as NK activating receptors (Jacobs *et al.*, 2005). In this study, incubation of PBMCs in the presence or absence of

IL-2 with mAbs against these molecules did not induce CD69 expression on NK cells. In contrast, activation of NK cells was detected when PBMCs were incubated in the presence of IL-2 with the NKG2D ligand MICA/Fc chimera. NKG2D binding by MICA/Fc chimera synergised strongly with IL-2 for activation of NK cells. Agreeing with this result, Andre *et al.* also reported a differential ability of anti-NKG2D mAb and MICA/Fc chimera to activate NK cells (Andre *et al.*, 2004). The failure to activate NK cells using mAbs against NKG2C, NKG2D or CD161 suggests that NK cells require additional stimulation to induce full activation; or perhaps that the epitopes these mAbs bind to are not activatory.

Interestingly, I obtained some evidence to suggest that CD81 cross-linking inhibited the up-regulation of CD69 expression on NK cells in response to stimulation with MICA/Fc chimera in the presence of IL-2; and importantly this effect was not detected when the anti-CD81 mAb was replaced with isotype-matched control IgG1 (Fig. 4.8). Unlike the effect observed on other responses (Fig. 4.4, 4.5, 4.10 and 4.11), this effect thus appeared to be CD81-specific. Since CD81 cross-linking did not alter the up-regulation of CD69 on NK cells in response to IL-2, the inhibitory effect of CD81 appears to be specific to the NK cell response to NKG2D ligation by the MICA/Fc chimera. Supporting this result, up-regulation of CD56 expression on NK cells induced by the MICA/Fc chimera was also inhibited by CD81 cross-linking (Fig. 4.9), whereas CD81 cross-linking did not specifically inhibit the IL-2-induced up-regulation of CD56 expression on NK cells (Fig. 4.11-E). Up-regulation of CD56 expression is a unique phenomenon that occurs during NK cell activation by certain stimuli. In this study, up-regulation of CD56 expression was observed when NK cells were stimulated with IL-2, IL12, IL-15 and IL-18. In contrast, stimulation of NK cells by CD16 cross-linking did not alter the expression of CD56 (data not shown). It could be speculated that the

change in CD56 expression requires signalling mediated by cytokines. Thus NKG2D ligation by the MICA/Fc chimera may function with IL-2 to activate NK cells through signalling pathways that are distinct from but complementary to those initiated by IL-2. It is assumed that CD81 inhibits the effect of NKG2D signalling.

Further investigation is required to confirm the effect of CD81 on the NK cell response to NKG2D ligation, and to understand the mechanism by which CD81 ligation inhibits NK cell activation in response to this stimulus. There are several issues I would have liked to address in further experiments. Firstly, it would have been of interest to confirm whether the effect I observed in this study could be reproduced using purified NK cells rather than total PBMCs. Andre *et al.* reported that activation of NK cells using NKG2D ligands could only be observed using polyclonal activated NK cells or NK cells within PBMCs, but not resting purified NK cells. It is not clear whether human NK cells require other cells types or cytokines to initiate full activation through NKG2D. Thus investigation of the effect of CD81 cross-linking on the response of purified NK cells to NKG2D ligation may not be possible. Secondly, the response of NK cells to a range of different concentrations of the MICA/Fc chimera in the presence of IL-2 should have been investigated, and the effect of CD81 ligation on the dose response curve tested (together with the effect of control IgG1 Abs) to assure the specificity of the effect observed, and determine its magnitude. Thirdly, the effect on different NK cell activities which NKG2D may stimulate would have been of interest to investigate. IFN-γ is a useful readout to determine NK activation, however, IFN-γ secretion by NK cells was not found to be enhanced by stimulation with the MICA/Fc chimera in the presence of IL-2. Several other studies also failed to detect IFN-γ production from PBMCs or purified NK cells in response to NKG2D ligation by anti-NKG2 mAbs or

NKG2D ligands (Andre *et al.*, 2004; Billadeau *et al.*, 2003). In contrast, NKG2D ligation by MICA/Fc chimera was found to induce IFN-γ production from polyclonally activated human NK cells (Andre *et al.*, 2004), and binding of another NKG2D ligand, ULBPs, also triggered IFN-γ secretion from cultured NK cells in the presence of IL-12 (Sutherland *et al.*, 2002). It is not clear why NKG2D ligation does not induce IFN-γ production from fresh PBMCs. In the future, it would be of interest to carry out experiments to investigate whether IL-12 enhances IFN-γ production by NK cells in response to NKG2D ligation, and if so, whether CD81 cross-linking can modulate this NK response. Measurement of cytotoxicity is frequently used as a readout for NK cell activity. However, careful choice of target cells is required when the effect of CD81 cross-linking on NK cytotoxic activity in response to NKG2D is analysed. Billadeau *et al.* and Pende *et al.* reported that NKG2D triggers cytotoxicity in human NK cells using murine mastocytoma P815 cells in redirected killing assays (Billadeau *et al.*, 2003; Pende *et al.*, 2001). Recently, it has been reported that the spontaneous NK-mediated killing of these cells is partly dependent on NKp46 engagement by murine cell ligands expressed on the P815 cells (Sivori *et al.*, 1999) and that blocking of NKp46 on NK cells can inhibit the ability of anti-NKG2D mAb to trigger NK-mediated killing of P815 cells (Andre *et al.*, 2004). These results imply that the induction of cytotoxicity by NKG2D requires the engagement of other triggering NK receptors. Altogether, further studies are required to understand the mechanism by which NKG2D ligands trigger full activation of NK cells, and that by which CD81 inhibits the activation of NK cells initiated by MICA/Fc chimera ligation.

In summary, the results obtained in this chapter contrast with results reported in the literature, and suggest that the effect of anti-CD81 mAbs on the response of NK cells to

CD16 cross-linking or cytokines may be Fc receptor-mediated. However, other results obtained suggest that mAb cross-linking of CD81 on NK cells may specifically inhibit their activation in response to NKG2D ligation. Had time permitted, further studies would have been done to extend understanding of the role of CD81 in NK activation in response to NKG2D ligation. Moreover, it would have been of interest to address whether the inhibitory effect of anti-CD81 mAbs on NK cells could be mimicked by HCV E2 protein binding to CD81.

Chapter 5 Role of CD81 on human and murine DCs in response to different stimuli

5.1. Introduction

Continuing the theme of investigating the role(s) of CD81 expression on different immune system cell types in modulating their activation/functions, in this chapter I went on to explore the involvement of CD81 in the response of DCs to different activating stimuli. Again, the long-term goal of this work was to gain insight into the potential for interaction of the HCV E2 glycoprotein with CD81 to modulate DC activity in infected individuals.

DCs are specialised APCs that play a central role in the initiation and direction of immune responses. DCs capture and process antigens in the periphery, and migrate into the lymphoid tissues to present antigens to T cells in a MHC-restricted manner. DCs are equipped with machinery not only for presenting endogenous and exogenous antigens through MHC class I and II molecules respectively but also for cross-presenting exogenous antigen through MHC class I molecules (den Haan *et al.*, 2000; Shen *et al.*, 1997). DCs are also involved in the maintenance of central and peripheral tolerance. Upon activation, DCs up-regulate expression of co-stimulatory molecules that are required for effective interaction with T cells, and also secrete various cytokines and chemokines to augment the innate and adaptive immune responses. Cytokines secreted by DCs include IL-12, IL-10 and IL-15 as well as other cytokines that promote an inflammatory response and the recruitment of activated cells to the site of infection. The production of IL-12 is particularly important due to its ability to induce Th1 responses in T cells. DCs are also an important source of type I IFNs, cytokines that have both direct anti-viral activity and mediate multiple immunomodulatory effects (Tough, 2004). Human DCs can be derived from several different lineages of precursor cells, including myeloid and plasmacytoid cells. Human myeloid DCs express high level of CD11c and

can be further divided into subtypes on the basis of expression of surface markers such as BDCA-1 and BDCA-3 (Dzionek *et al.*, 2000; MacDonald *et al.*, 2002). Myeloid DCs mediate antigen capture and processing very efficiently, and after receipt of appropriate maturation stimuli, they can act as highly potent APCs. Human plasmacytoid DCs express CD123 and BDCA-2, but lack CD11c expression (Dzionek *et al.*, 2001). They mediate antigen uptake and presentation relatively poorly, but are the major type I IFN producers in many viral infections, playing an important role in Th1 polarisation of responses (Asselin-Paturel *et al.*, 2001; Cella *et al.*, 2000; Kadowaki *et al.*, 2000). Since circulating blood DCs are heterogeneous and are difficult to obtain in large quantities, monocyte-derived DCs are often used as a surrogate for *in vivo* DC populations. Monocyte-derived DCs can be generated by culture of peripheral blood monocytes with GM-CSF, and their functions broadly resemble those of myeloid DCs.

Murine DCs can be also divided into $CD11c^{high}$ myeloid DCs and $CD11c^{low}$ plasmacytoid DCs. The conventional ($CD11c^{high}$) DC subset can be further subdivided on the basis of expression of CD4 and CD8. Hence at least four subtypes of DCs can be distinguished in the spleen of mice: $CD11c^{high}CD4^{-}CD8^{+}$, $CD11c^{high}CD4^{+}CD8^{-}$ and $CD11c^{high}CD4^{-}CD8^{-}$ conventional DCs (Vremec *et al.*, 2000) and $CD11c^{low}B220^{+}DX5^{-}$ plasmacytoid DCs (Nakano *et al.*, 2001). The different subtypes of $CD11c^{high}$ DCs share a common ability to present antigens efficiently to T cells, however only $CD8^{+}$ DCs are able to cross-present exogenous antigens on MHC class I molecules (den Haan *et al.*, 2000). $CD8^{+}$ DCs typically tend to induce a Th1-biased T cell responses, whereas $CD4^{+}$ DCs tend to induce Th2-biased T cell responses (Moser & Murphy, 2000). As in human, resting murine plasmacytoid DCs are relatively inefficient APCs, but act as an important source of type I IFN production during infections (Asselin-Paturel *et al.*, 2001). It is also possible to generate large quantities of murine DCs by culturing bone

marrow cells in the presence of cytokines *in vitro*. Bone marrow-derived DCs generated by culture with GM-CSF and IL-4 resemble conventional DCs more closely than plasmacytoid DCs.

The functional properties of DCs are determined not only by their lineage, but also by the maturation and activation signals they receive. DCs are highly responsive to stimuli such as microbial infection and tissue damage, and their maturation is further driven by interaction with T cells (Reis e Sousa, 2001). Interestingly, not all signals induce equivalent DC activation, thus different stimulatory signals are translated by DCs to yield different types of immune response. DCs are equipped with series of pattern-recognition receptors which recognise pathogen-associated molecular patterns (PAMPs) carried by potential invaders. TLRs are one family of pattern-recognition receptors. Eleven members of the TLR family have been identified (TLR1-11) and each member is thought to recognise a distinct subset of PAMPs, e.g. TLR5 recognises flagellin and TLR9 recognises CpG DNA (Takeda & Akira, 2004; Yarovinsky *et al.*, 2005). Human *ex vivo* immature $CD11c^+$ DCs express all TLRs apart from TLR9, while human plasmacytoid DCs predominantly express TLRs1, 6, 7 and 9 (Reis e Sousa, 2004). Human monocyte-derived DCs express TLRs1, 2, 3, 4 and 5 (Reis e Sousa, 2004). Mouse $CD4^+$, $CD8^+$ DCs and plasmacytoid DCs express TLRs1-9 with the exception being that $CD8^+$ DCs lack TLR7 expression (Reis e Sousa, 2004). The plasmacytoid DC subset expresses particularly high levels of TLRs7 and 9, whilst TLR3 is expressed at high levels on $CD8^+$ DCs and TLRs5 and 6 on $CD4^+$ DCs.

The TLRs consist of: (1) an extracellular domain which recognises distinct PAMPs, and (2) a cytoplasmic signalling domain that shows high similarity to that of the IL-1 receptor family, called the Toll/IL-1 receptor (TIR) domain. TLR4 is known to form a

LPS recognition complex with CD14 and MD2 (Medzhitov *et al.*, 1997; Shimazu *et al.*, 1999; Wright *et al.*, 1990). MD2 is associated with TLR4 and both molecules are indispensable for LPS responses (Shimazu *et al.*, 1999). In contrast, mice without CD14 still show responses against LPS (Haziot *et al.*, 1996), indicating that CD14 plays a role in enhancing the interaction of LPS with TLR4-MD2 and amplifying subsequent signalling (da Silva Correia *et al.*, 2001). As shown in Fig. 5.1, engagement of TLR4 with LPS triggers the recruitment of adaptor proteins MyD88 and TRIF and activation of intracellular signalling leading to NF-κB and MAPK activation and IRF-3 activation. This in turn results in the induction of proinflammatory cytokines such as TNF-α, IL-6 and IL-12 and DC maturation which is associated with up-regulation of co-stimulatory molecules such as CD80, CD86 and CD40. IRF-3 activation leads to the induction of type I IFN production.

TLR3 recognises double-stranded (ds) RNA (Alexopoulou *et al.*, 2001). A synthetic analogue of dsRNA, poly(I:C), can also bind TLR3. The TIR domain of TLR3 recruits the adaptor protein TRIF, via which IRF-3 activation is signalled (Oshiumi *et al.*, 2003), as shown in Fig. 5.1-A. Cross-talk between DCs and T cells is mediated in part by interaction of CD40 on DCs with CD40 ligand on activated T cells. CD40 is known to play a key role in triggering DC to become potent T-cell activators. CD40 forms homotypic trimers. Following binding to CD40 ligand, CD40 recruits and activates TRAFs 2, 3, 5 and 6, which initiates activation of the NF-kB and MAPK signalling pathways, as shown in Fig. 5.1.

CD81 is expressed on human myeloid and plasmacytoid DCs, as shown in the previous chapter. CD81 is also known to be expressed on mouse DCs (Maecker *et al.*, 2000). Despite the presence of CD81 on DCs, its role on these cells is not clear. Interestingly, it

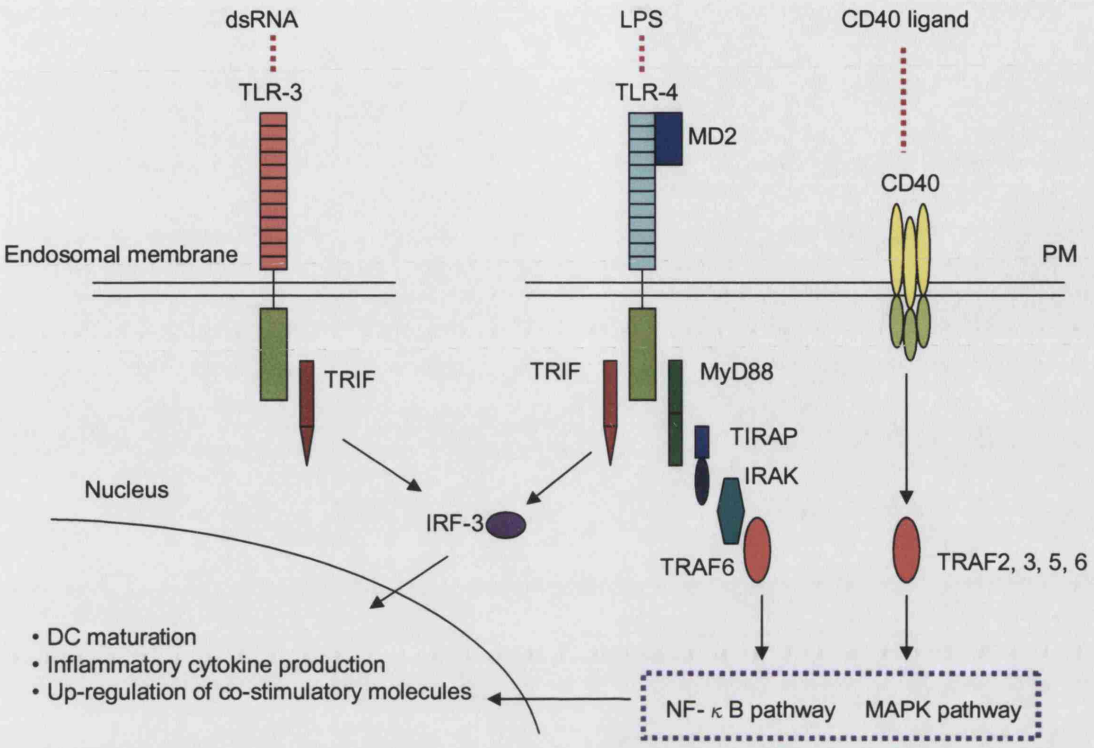


Figure 5.1. Signalling pathways involved in the activation of DCs.

Signalling pathways involved in the activation of DCs are shown. Receptors via which DC activation and maturation can be triggered include Toll-like receptors (TLRs) and CD40. TLR4 and CD40 are expressed on the plasma membrane (PM) and they recognise LPS and CD40 ligand respectively. TLR3 is expressed on the endosomal membrane and recognises double-stranded (ds) RNA. TLR4 is associated with the Toll/IL-1 receptor (TIR) domain-containing adaptor proteins myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor-inducing IFN-β (TRIF). Upon LPS binding to TLR4, MyD88 recruits and activates IL-1 RI-associated protein kinase (IRAK) 4 and the tumour necrosis factor receptor-associated factor (TRAF) 6, which results in the activation of both NF-κB and MAPK signalling pathways. In parallel, TRIF activates translocation of IFN regulatory factor-3 (IRF-3) in to nucleus. Upon dsRNA binding to TLR3, TRIF is recruited to TLR3 leading to activation of IRF-3. After CD40 ligation, TRAFs 2, 3, 5 and 6 are recruited, leading to activation of both the NF-κB and MAPK signalling pathways.

has been reported that CD81 forms clusters with CD14 and TLR4 on monocytes when these cells are activated by LPS (Pfeiffer *et al.*, 2001). Although monocytes down-regulate expression of CD14 when they differentiate into monocyte-derived DCs (Engering *et al.*, 2002), it is possible that CD81 may associate with TLR4 upon LPS ligation on monocyte-derived DCs. Since CD81 cross-linking modulates the response of T cells to stimuli triggered through the receptor complex that CD81 associates with on these cells, I hypothesised that CD81 cross-linking on DCs may likewise modulate signalling triggered by the activation receptor complex(es) it associates with on these cells, including the TLR4 complex. One of the goals of the work in this chapter was thus to address the effect of CD81 cross-linking on the response of human monocyte-derived DCs to different activating stimuli including LPS.

As an alternative approach to understanding the role of CD81 on DCs, experiments were also carried out in this chapter with DCs from CD81 KO mice. As reviewed in the main introduction, a number of studies have characterised T and B cell responses in $CD81^{-/-}$ mice (Deng *et al.*, 2002; Deng *et al.*, 2000; Maecker & Levy, 1997; Maecker *et al.*, 1998; Miyazaki *et al.*, 1997), but activation and functions of DCs lacking CD81 expression have not been directly addressed. Thus, I initially characterised the number and subset composition of the spleen DC population in $CD81^{-/-}$ mice, and then investigated the response of bone marrow-derived DCs from CD81-deficient mice to various stimuli (LPS, poly(I:C) and an anti-CD40 mAb). Together, it was hoped that these studies would give more information about potential role(s) of CD81 in DC responses.

5.2. Results

5.2.1. Analysis of the expression of CD81 on human monocyte-derived DCs responding to activating stimuli

CD81 expression on human T and B cells is known to be down-regulated when these cells are stimulated by anti-CD3 and anti-CD28 mAbs or anti-IgM mAb and IL-4 respectively (Fritzsching *et al.*, 2002), and human NK cells also down-regulate CD81 expression upon activation (personal communication from Dr Nicholas Valiante). I thus explored whether CD81 expression on human DCs was altered when they were activated. Monocyte-derived DCs were generated by culturing monocytes in the presence of GM-CSF and IL-4. On day 6/7 of culture, more than 95 % of viable cells harvested expressed CD11c. The DCs produced in this way had an immature phenotype as indicated by the low % of cells expressing the maturation marker CD83 (routinely 20-40 %) and low level expression of co-stimulatory molecules (data not shown).

These monocyte-derived DCs were harvested and stimulated with LPS, and surface CD81 expression on these cells was monitored. Consistent with results shown in Fig. 3.5, CD81 was expressed on almost all monocyte-derived DCs prior to LPS stimulation. After a 3 hour incubation with LPS, the monocyte-derived DCs began to up-regulate expression of CD83, with further up-regulation being evident after overnight incubation (Fig. 5.2-A). In contrast, monocyte-derived DCs were found to down-regulate CD81 expression following LPS stimulation, with down-regulation occurring by 3 hours post-stimulation at the time point when up-regulation of CD83 expression started to be observed and CD81 expression still being reduced after overnight incubation (Fig. 5.2-B). Down-regulation of surface CD81 expression was also observed when DCs were stimulated with poly(I:C) or an anti-CD40 mAb (data not shown). However, these stimuli were not as potent as LPS, and the changes in surface marker expression (both

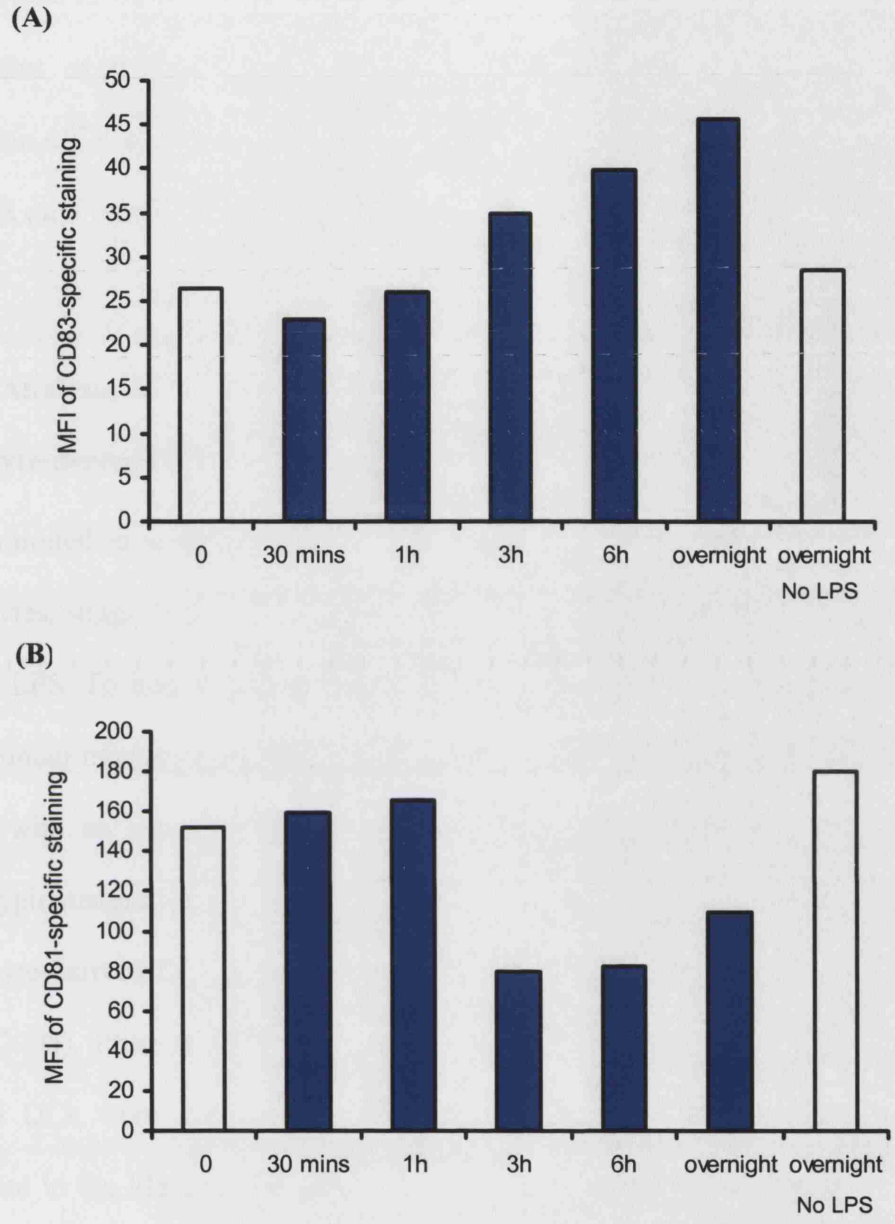


Figure 5.2. Effect of LPS stimulation on CD83 and CD81 expression on monocyte-derived DCs.

Monocyte-derived DCs were incubated for 30 minutes (mins), 1 hour (h), 3h, 6h or overnight with 300 ng/ml LPS (blue bars) or without LPS (No LPS; white bars) as a control. DCs were then stained with anti-CD83 or anti-CD81 mAbs, and analysed by flow cytometry. The results shown are the mean fluorescence intensity (MFI) of CD83 (A) or CD81 (B) -specific staining on DCs.

the increase in CD83 expression and the decrease in CD81 expression) were observed only after overnight incubation. Thus there seemed to be a correlation between activation of DCs and down-regulation of CD81 expression on these cells similar to that reported on T cells.

5.2.2. Analysis of the effect of CD81 cross-linking on the response of human monocyte-derived DCs to LPS

As mentioned in section 5.1, CD81 is found in association with CD14 and TLR4 on monocytes, suggesting the possibility that CD81 cross-linking may alter the response of DCs to LPS. To investigate the effect of CD81 cross-linking on the response of DCs to LPS, human monocyte-derived DCs were stimulated with LPS in plates that had been coated with an anti-CD81 mAb or isotype-matched (IgG1) control mAb, and their phenotypic maturation was analysed.

Monocyte-derived DCs express CD32 (FcgRII), which binds to IgG1 molecules (Banki *et al.*, 2003). Prior to the stimulation of cells in plates coated with mAbs, monocyte-derived DCs were thus treated with an anti-CD32 mAb to block Fc-receptors, as described in the Materials and Methods. It was confirmed that treatment with the anti-CD32 mAb did not activate DCs (data not shown). As shown in Fig. 5.3, LPS induced the phenotypic maturation of DCs in a dose-dependent manner. At high LPS doses, expression of the maturation marker CD83 was induced on almost all DCs. The co-stimulatory molecules CD40 and CD86 were up-regulated on DCs, as was the adhesion molecule CD54, CD95, and also MHC class I and II (not shown). The chemokine receptor CCR5 was down-regulated, whereas CCR7 was up-regulated. No difference was observed between the response of cells in control plates and those in plates coated

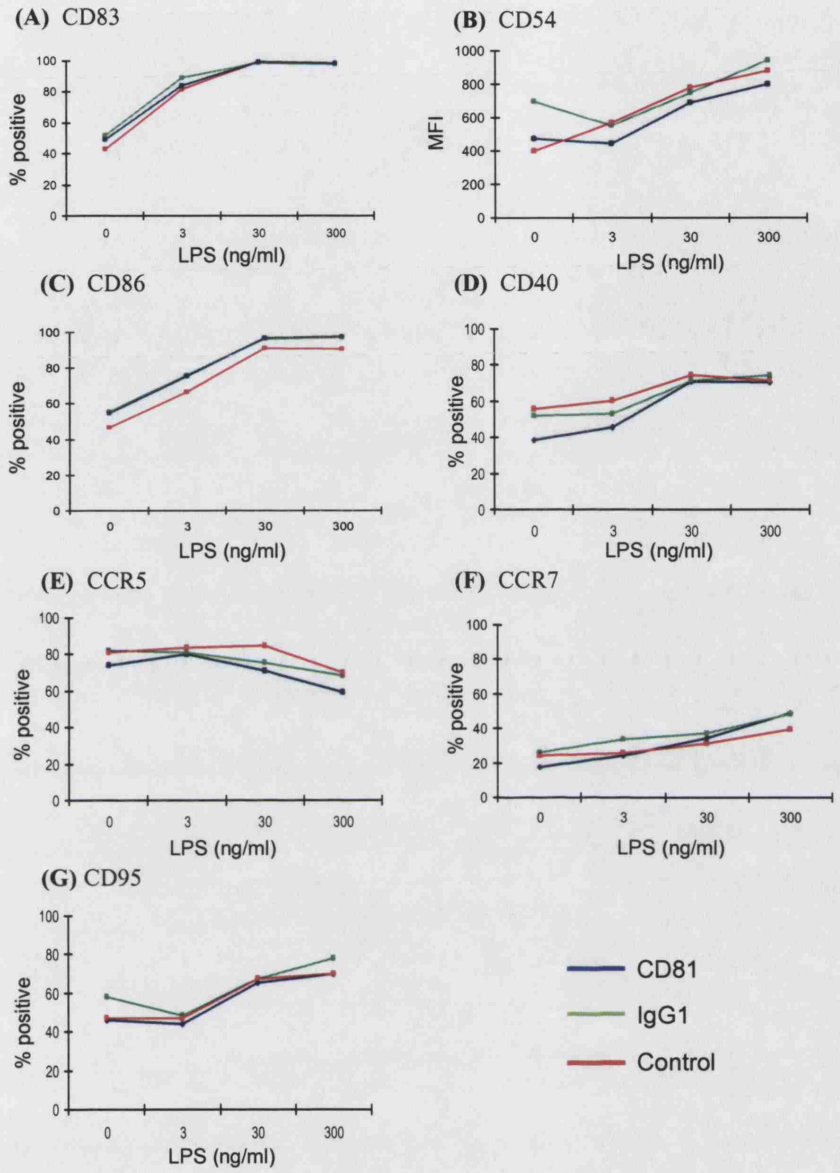


Figure 5.3. Analysis of the effect of CD81 cross-linking on the phenotypic activation of monocyte-derived DCs in response to LPS stimulation.

Monocyte derived DCs were stimulated with the indicated concentrations of LPS in control plates (Control), or plates coated with 10 µg/ml anti-CD81 mAb (JS-81 clone; CD81) or with an isotope-matched control mAb (IgG1). After overnight incubation, cells were stained with antibodies against CD83 (A), CD54 (B), CD86 (C), CD40 (D), CCR5 (E), CCR7 (F), or CD95 (G) and analysed by flow cytometry. The results are expressed as either the % of cells expressing the indicated surface markers or the mean fluorescence intensity (MFI) of mAb staining, and are representative of findings made in 10 independent experiments using cells from different donors.

with a control IgG1 mAb. In addition, CD81 cross-linking by anti-CD81 mAb also did not alter the phenotypic response of DCs to LPS.

The activation of DCs in response to LPS stimulation was also analysed at a functional level, by analysing production of variety of pro-inflammatory cytokines (IL-1, IL-6, TNF-α, IL-12 and IL-8), and also the immunosuppressive cytokine IL-10. Upon LPS stimulation, monocyte-derived DCs produced IL-1β, IL-6, IL-12 p40/p70, TNF-α, IL-10 and IL-8 in a dose-dependent manner; IL-12 p40, TNF-α, IL-10 and IL-6 production is shown in Fig. 5.4. Again, however, cross-linking of CD81 did not alter the response observed: both the pattern of cytokines produced and the magnitude of the response were indistinguishable from those of control or control mAb stimulated DCs.

CD81 cross-linking was thus not found to alter the response of DCs to LPS, at least as assessed by phenotypic maturation and cytokine production.

5.2.3. Analysis of the effect of CD81 cross-linking on the response of monocyte-derived DCs to poly(I:C)

Monocyte-derived DCs also express TLR3, which activates cells via a MyD88-independent signalling pathway. Further experiments were carried out to investigate whether CD81 cross-linking alters the response of monocyte-derived DCs to poly(I:C), a TLR3 ligand.

Poly(I:C) induced DC maturation in a dose-dependent manner (Fig. 5.5). It was a less potent stimulus for DC maturation than LPS; nonetheless at the highest concentrations used, poly(I:C) induced expression of the maturation marker CD83 on the majority of DCs. Expression of MHC class II, CD95, CD86, CD40 and CCR7 was also up-regulated, while CCR5 expression was slightly down-regulated. However, CD81 cross-

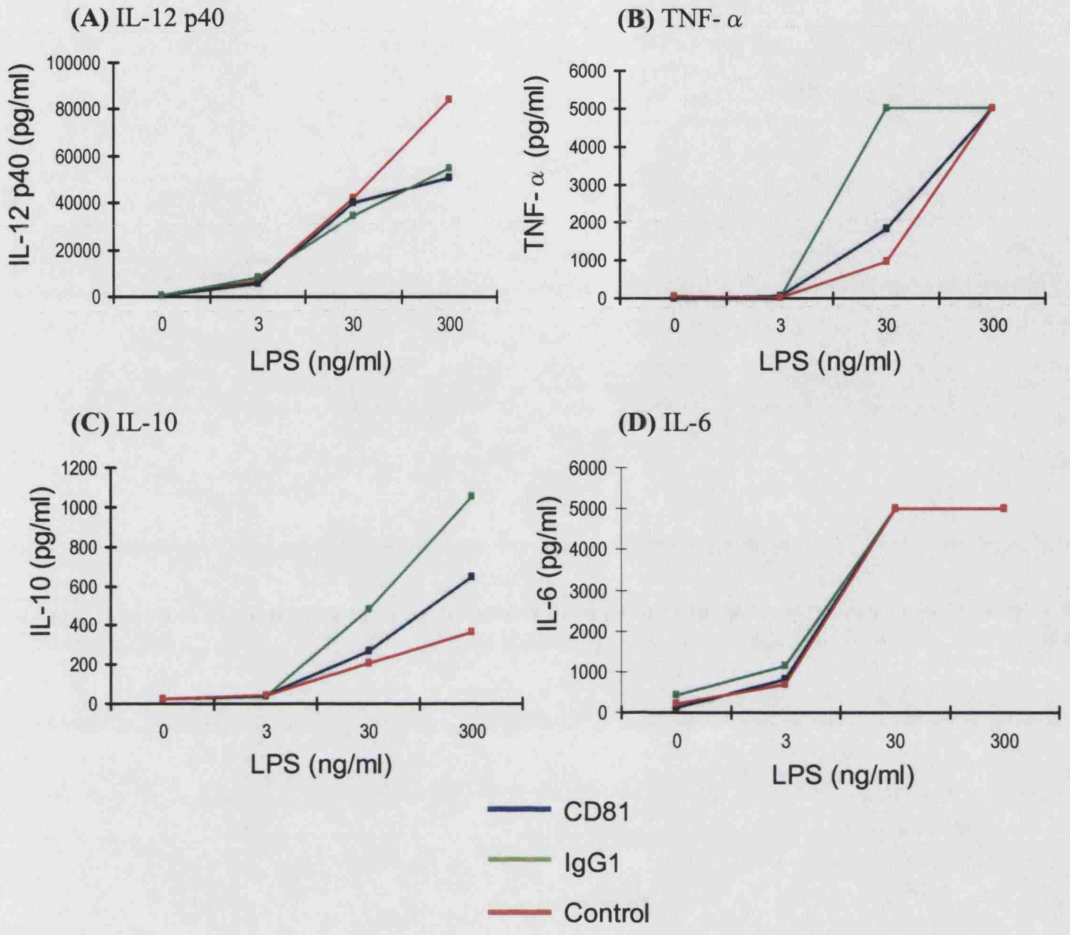


Figure 5.4. Analysis of the effect of CD81 cross-linking on cytokine production by monocyte-derived DCs in response to LPS.

Monocyte-derived DCs were stimulated with the indicated concentrations of LPS in control plates (Control), or plates coated with 10μg/ml anti-CD81 mAb (JS-81 clone; CD81) or with an isotope-matched control mAb (IgG1). After overnight incubation, supernatants were harvested and cytokine contents were measured. IL-12 p40 (A) was measured by ELISA, and TNF-α (B), IL-10 (C) and IL-6 (D) were measured by CBA assay. The results shown are representative of findings made in 5 independent experiments using cells from different donors.

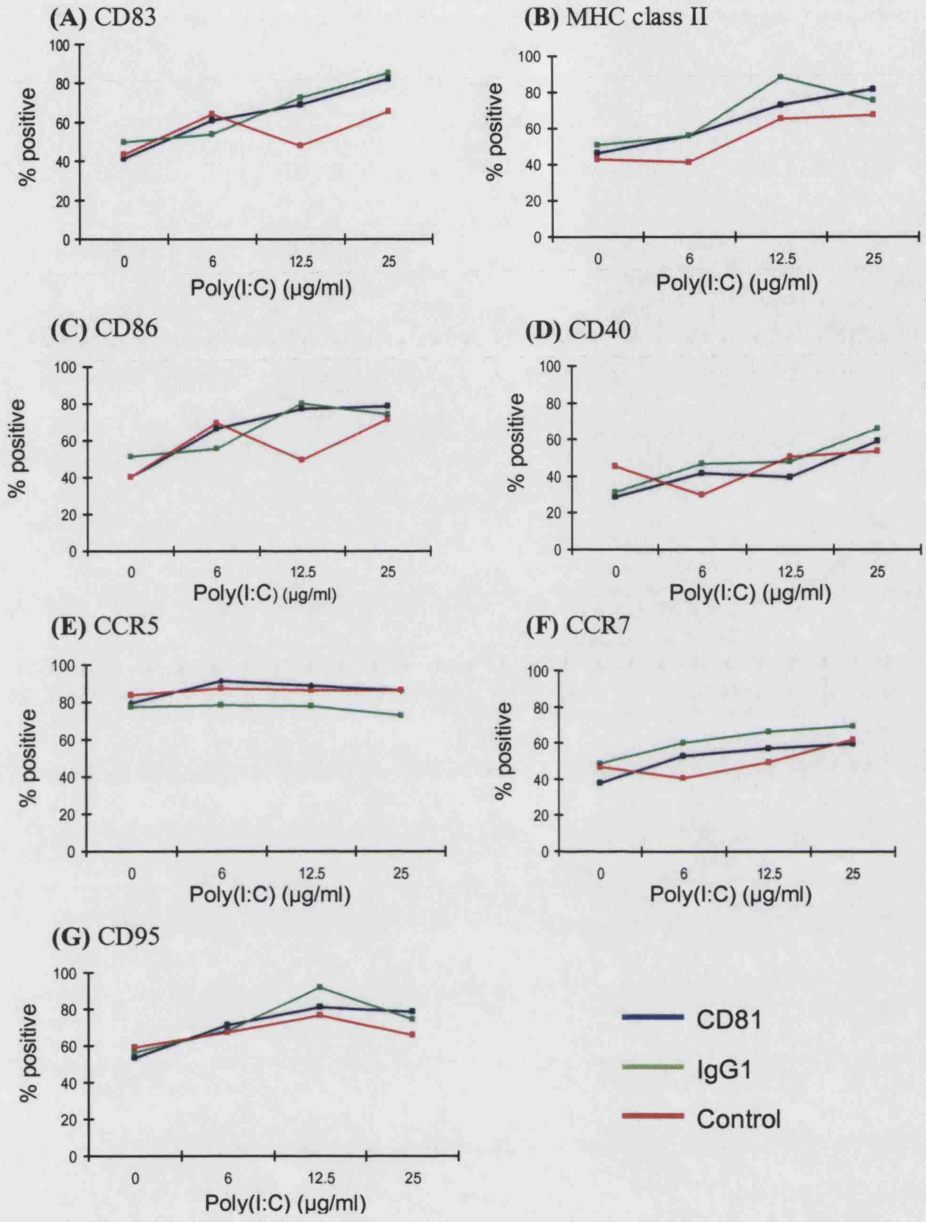


Figure 5.5. Analysis of the effect of CD81 cross-linking on the phenotypic activation of monocyte-derived DCs in response to poly(I:C) stimulation.

Monocyte-derived DCs were stimulated with the indicated concentrations of poly(I:C) in control plates (Control), or plates coated with 10µg/ml of anti-CD81 mAb (CD81) or an isotope-matched control mAb (IgG1). After overnight incubation, cells were stained with antibodies against CD83 (A), MHC class II (B), CD86 (C), CD40 (D), CCR5 (E), CCR7 (F), or CD95 (G) and analysed by flow cytometry. The results are expressed as the % of cells expressing the indicated surface markers and are representative of findings made in 2 experiments using cells from different donors.

linking by anti-CD81 mAb did not alter the phenotypic activation of monocyte-derived DCs in response to poly(I:C).

Cytokine production by poly(I:C) stimulated DCs was also assessed (Fig. 5.6). This TLR3 ligand stimulated production of many of the same cytokines as LPS, including IL-12 p70, IL-6 and IL-8, which were produced in a dose-dependent manner. In contrast to cells stimulated with LPS, monocyte-derived DCs stimulated with poly(I:C) did not produce much TNF-α, IL-10 or IL-1β at the poly(I:C) doses I used in this study. Once again, however, cross-linking of CD81 did not alter the response observed: both the pattern of cytokines produced and the magnitude of the response were indistinguishable from those of control or control mAb stimulated DCs.

CD81 cross-linking was thus not found to alter the response of DCs to poly(I:C), at least as assessed by phenotypic maturation and cytokine production.

5.2.4. The effect of CD81 cross-linking on the response of monocyte-derived DCs to stimulation with an anti-CD40 mAb

Another pathway by which DC maturation can be induced is via interaction of CD40 on the DC surface with CD154 (CD40 ligand) on $CD4^+$ T cells. This can be mimicked by cross-linking of CD40 with an anti-CD40 mAb (O'Sullivan & Thomas, 2003; Watanabe *et al.*, 2003). To analyse whether CD81 cross-linking alters the activation of DCs through CD40 signalling, monocyte-derived DCs were stimulated in plates coated with an anti-CD40 mAb in combination with anti-CD81 mAb or isotype-matched control mAb IgG1, and their phenotypic and functional activation was assessed.

Unlike LPS and poly(I:C), cross-linking of CD40 by an anti-CD40 mAb did not induce phenotypic DC maturation of DCs under the experimental conditions I used (Fig. 5.7). There was no increase in the % of cells expressing CD83, and none of the other surface

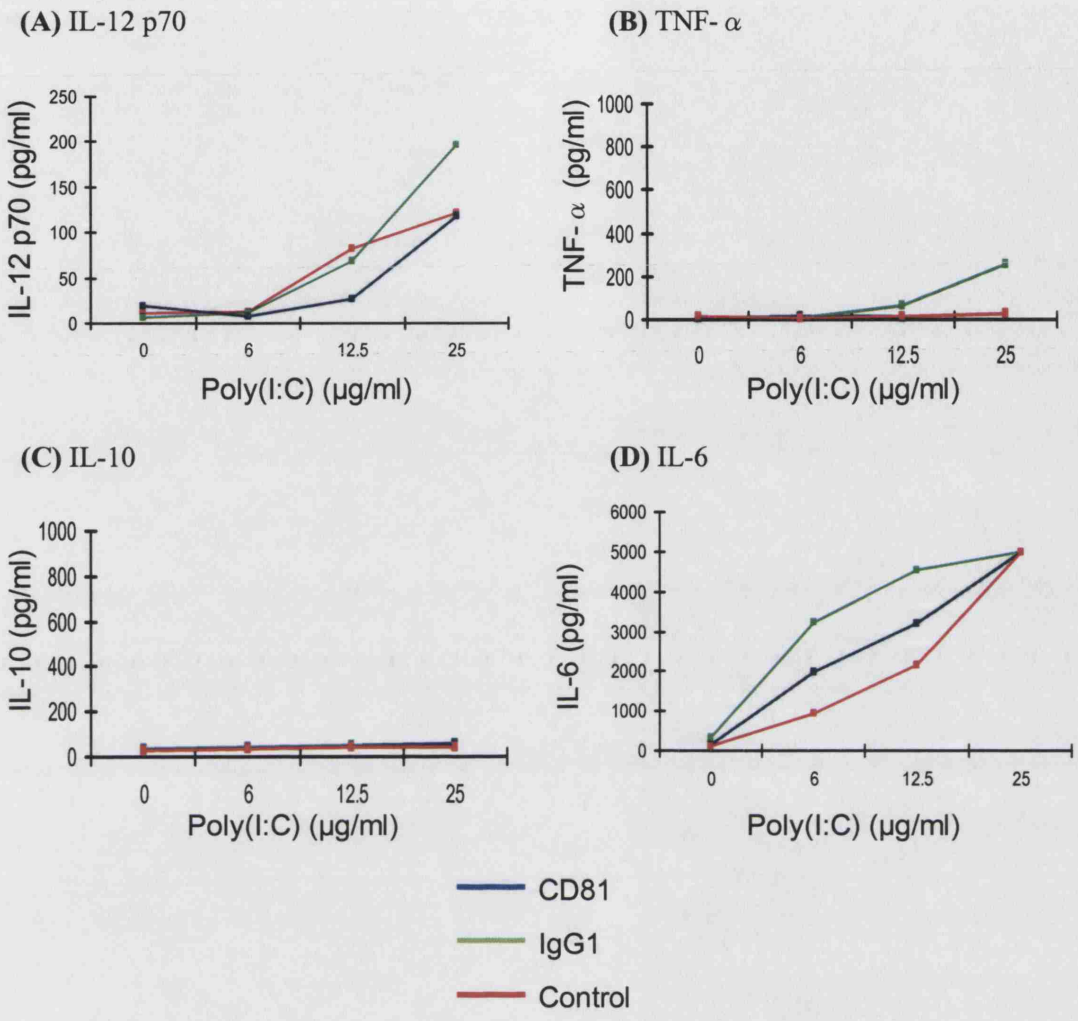


Figure 5.6. Analysis of the effect of the CD81 cross-linking on cytokine production by monocyte-derived DCs in response to poly(I:C).

Monocyte-derived DCs were stimulated with the indicated concentrations of poly(I:C) in control plates (Control), or plates coated with 10µg/ml anti-CD81 mAb (JS-81 clone; CD81) or with an isotope-matched control mAb (IgG1). After overnight incubation, supernatants were harvested and cytokine contents were measured. IL-12 p70 (A), TNF-α (B), IL-10 (C) and IL-6 (D) were measured by CBA assay. The results shown are representative of findings made in 2 independent experiments using cells from different donors.

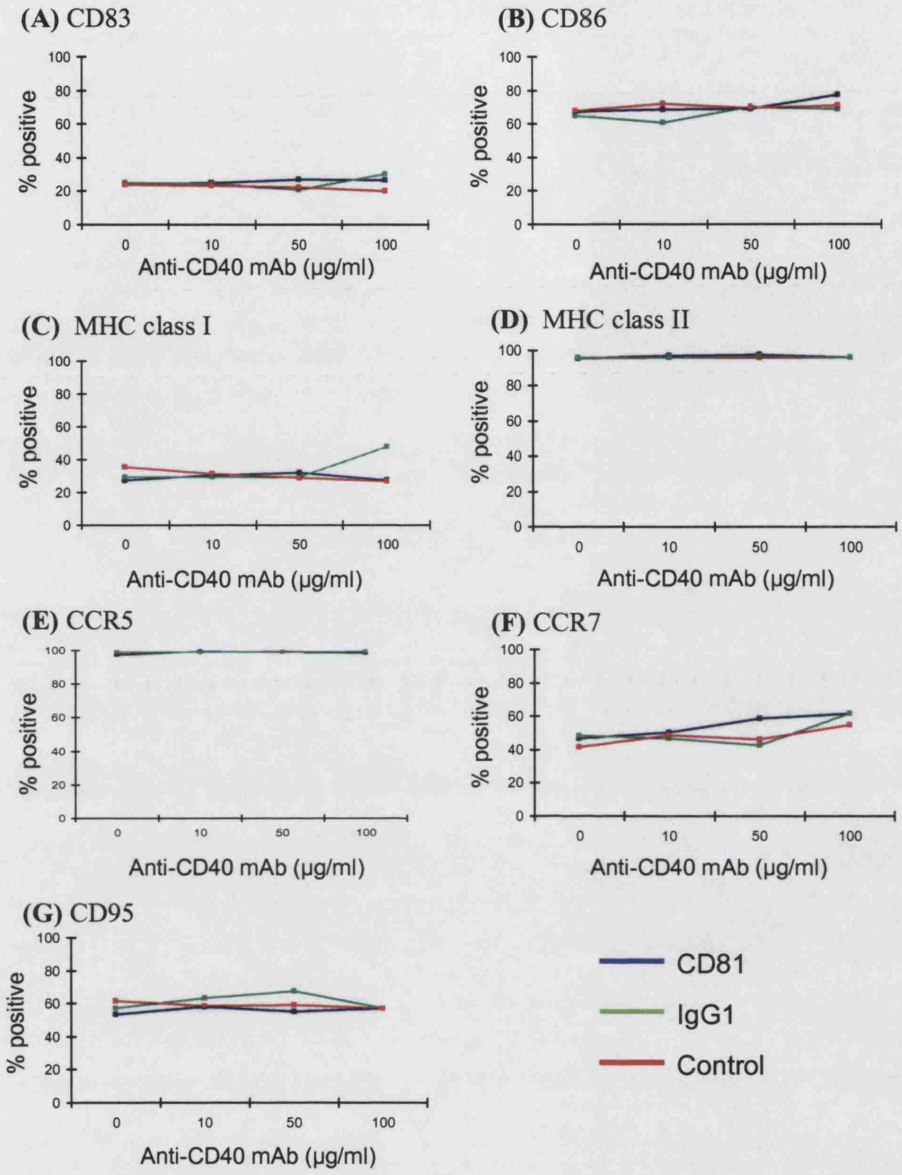


Figure 5.7. Analysis of the effect of CD81 cross-linking on surface marker expression on monocyte-derived DCs stimulated with an anti-CD40 mAb.

Monocyte-derived DCs were stimulated in plates coated with the indicated concentrations of anti-CD40 mAb either alone (Control) or in combination with 10µg/ml of anti-CD81 mAb (CD81) or an isotope control mAb (IgG1). After overnight incubation, cells were stained with antibodies against CD83 (A), CD86 (B), HLA-ABC (C), HLA-DR (D), CCR5 (E), CCR7 (F) or CD95 (G), and analysed by flow cytometry. The results are expressed as the % of cells expressing the indicated surface markers, and are representative of findings made in 4 independent experiments using cells from different donors.

markers evaluated was up-regulated by anti-CD40 stimulation. The % of DCs expressing each marker is shown in Fig. 5.7, but the MFI of staining with mAbs to these molecules also did not change after anti-CD40 mAb ligation (data not shown). As also illustrated in Fig. 5.7, CD81 cross-linking did not induce any alteration in surface marker expression on DCs in response to CD40 cross-linking.

Although stimulation of monocyte-derived DCs with an anti-CD40 mAb did not induce any detectable phenotypic change in the cells, they did produce cytokines in response to CD40 cross-linking (Fig. 5.8). Unlike when DCs were stimulated with LPS or poly(I:C), IL-12 was the dominant cytokine produced by monocyte-derived DCs stimulated with an anti-CD40 mAb; dose-dependent production of IL-12 p40 was observed. On the other hand, TNF-α, IL-10, IL-6, IL-8, and IL-1β were hardly produced by monocyte-derived DCs stimulated with an anti-CD40 mAb. Once again, cross-linking of CD81 was not found to alter the pattern of cytokines produced by DCs in response to CD40 cross-linking, or the magnitude of the response. CD81 cross-linking was thus not found to alter the response of DCs to an anti-CD40 mAb, at least as assessed by phenotypic maturation and cytokine production.

In summary, CD81 cross-linking was not found to have any effect on the response (as assessed by phenotypic maturation and cytokine production) of monocyte-derived DCs to stimulation with LPS, poly(I:C) or an anti-CD40 mAb. This could be because CD81 cross-linking does not modulate any of responses assessed. Alternatively, it could be because the mAb cross-linking approach was not adequately mimicking CD81 cross-linking by natural ligand(s). The anti-CD81 mAb used was able to modulate T cell responses (Chapter 4) and did bind to monocyte-derived DCs (Chapter 3 and Fig. 5.2). However, the MFI of CD81 staining on DCs was lower than on T cells. Thus it is

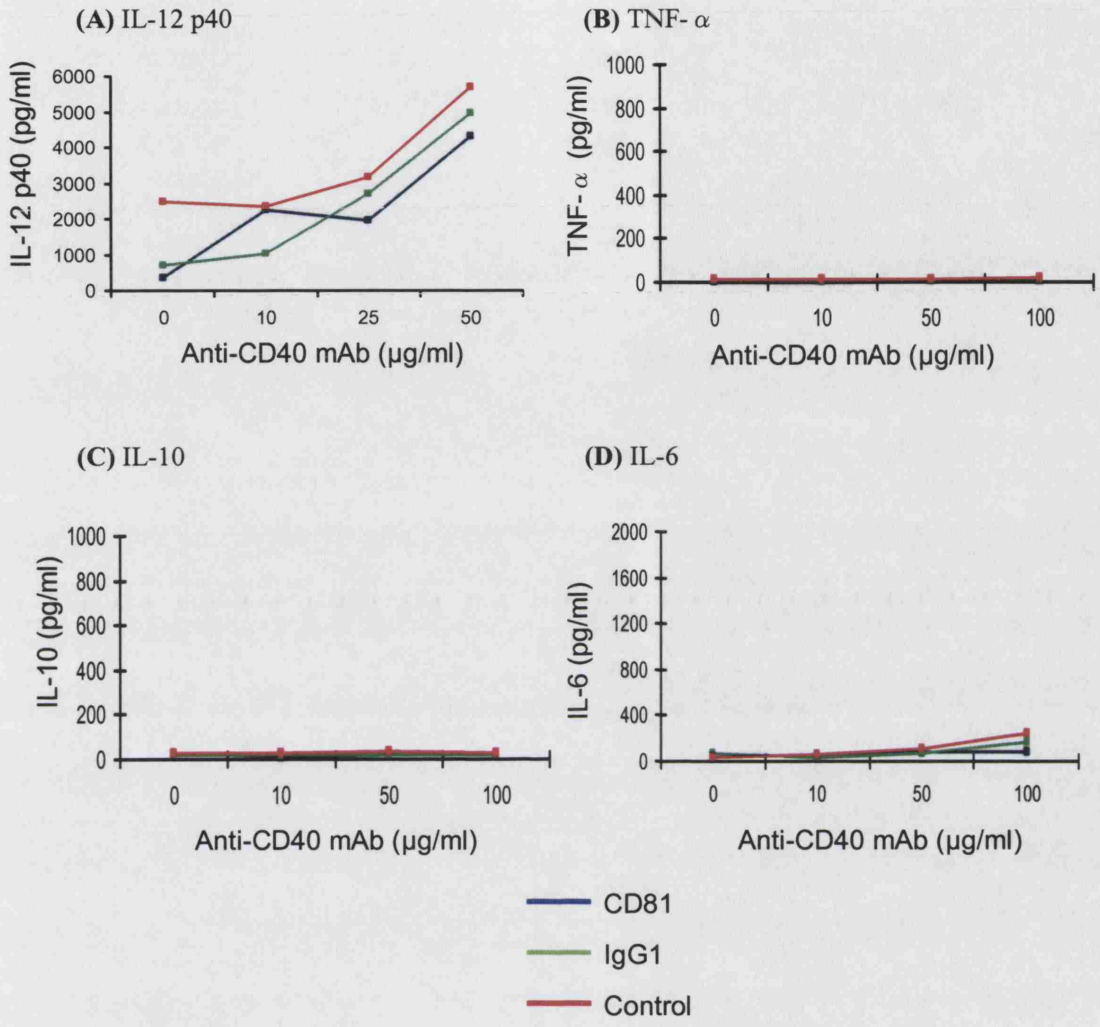


Figure 5.8. Analysis of the effect of CD81 cross-linking on cytokine production by monocyte-derived DCs stimulated by an anti-CD40 mAb.

Monocyte-derived DCs were stimulated in plates coated with the indicated concentrations of anti CD40 mAb either alone (Control) or in combination with 10µg/ml of anti-CD81 mAb (CD81) or an isotope control mAb (IgG1). After overnight incubation, supernatants were harvested and cytokine contents were measured. IL-12 p40 (A) was measured by ELISA, and TNF-α (B), IL-10 (C) and IL-6 (D) were measured by CBA assay. The data shown are representative of findings made in 4 independent experiments using cells from different donors.

plausible that the anti-CD81 mAb did not bind to CD81 on DCs as well as to that on T cells, or that the level of expression of CD81 on DCs was too low to enable cross-linking by mAbs in a plate-bound system. Thus I took an alternative approach to assessing the role of CD81 on DCs.

5.2.5. Screening of CD81 KO mice

An alternative way to investigate the role of CD81 on DCs is to analyse DCs from CD81 KO ($CD81^{-/-}$) mice. CD81-deficient mice were obtained from Dr Raif Geha (Harvard Medical School, Boston, USA). These mice had been generated by replacing exons 2-8 of the CD81 gene, which include most of the coding regions, by a neomycin resistance gene (Fig. 5.9-A) (Tsitsikov *et al.*, 1997). Dr Geha originally supplied a small number of heterozygous ($CD81^{+/-}$) mice. These were bred with wild type (WT; $CD81^{+/+}$) C57BL/6 females, and the offspring were re-derived under sterile conditions and fostered onto SPF mothers.

To enable $CD81^{+/+}$ C57BL/6 offspring to be distinguished from the $CD81^{+/-}$ offspring, a PCR screening method was set up. A primer pair was designed to amplify a 328 bp fragment of the inserted neomycin resistance gene. DNA from $CD81^{+/+}$ mice was not amplified by the neomycin resistance gene primers (Fig. 5.9-B). On the other hand, fragments of the expected size were amplified from DNA of $CD81^{+/-}$ mice by the neomycin resistance gene primers. Heterozygous mice identified in this way were bred together to generate homozygous $CD81^{-/-}$ mice.

To enable $CD81^{-/-}$ KO mice to be distinguished from $CD81^{+/-}$ or $CD81^{+/+}$ mice, surface expression of CD81 on PBLs was analysed by flow cytometry. PBLs from $CD81^{-/-}$ KO mice did not express CD81 whereas PBLs from $CD81^{+/-}$ KO mice and $CD81^{+/+}$ mice showed CD81 expression (Fig. 5.10).

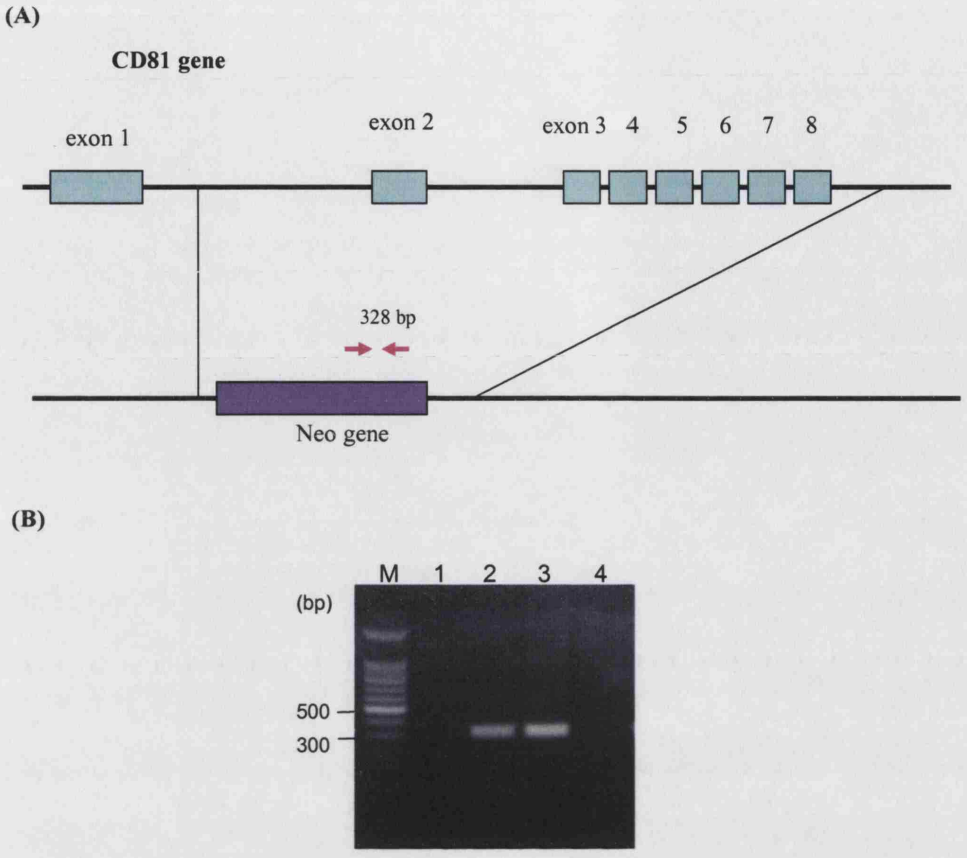


Figure 5.9. PCR method for identification of mice carrying a disrupted (KO) CD81 gene.

(A) The structure of the murine CD81 gene and the target construct used by Tsitsikov *et al.*, (1997) to generate CD81 KO mice are shown. The 16 kb murine CD81 gene consists of eight exons. Exons 2-8 were replaced by a neomycin resistance (Neo) gene by homologous recombination in the generation of CD81 knock-out mice. A PCR screening method was set up to allow wild-type (WT) mice and mice heterozygous /homozygous for the CD81 knockout (KO) mutation to be distinguished. This involved the use of a primer pair designed to amplify a 328 bp fragment of the Neo gene inserted during disruption of the CD81 gene (pink arrows).

(B) The gel pictured shows the PCR products obtained when DNA from a WT mouse (lane 1) and two heterozygous/homozygous CD81 KO mice (lanes 2 and 3) was tested by PCR with the primers shown in (A). A 100 bp DNA ladder (M) was run on the gel in parallel with the PCR products. A control PCR reaction without DNA was also included (lane 4).

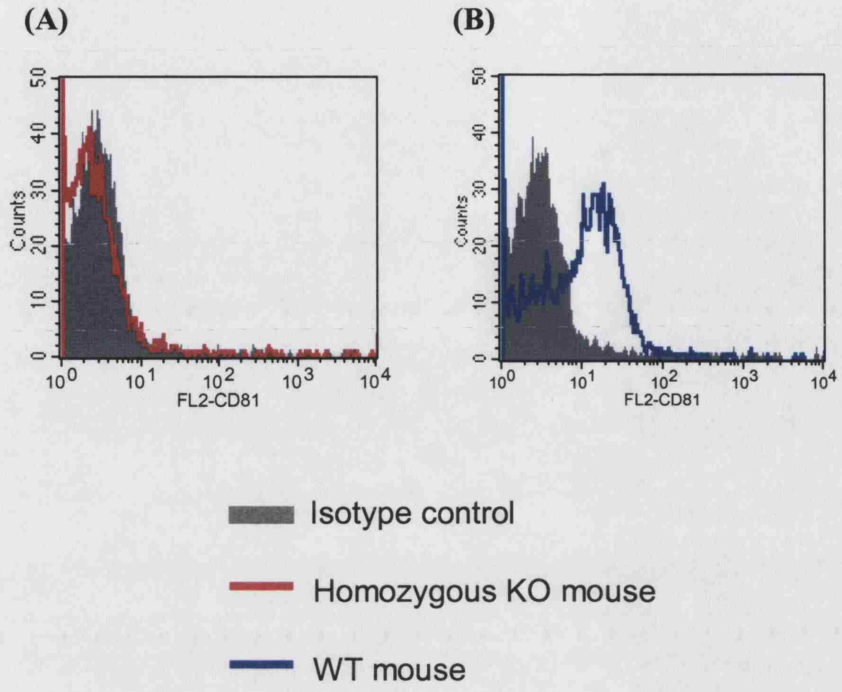


Figure 5.10. Use of a FACS-based method for detecting surface expression of CD81 to screen for CD81 KO mice.

Wild-type (WT) and heterozygous mice were distinguished from CD81 KO (KO) mice by flow cytometric analysis of CD81 expression on PBLs. PBLs were stained with a PE-conjugated anti-mouse CD81 mAb or an isotype-matched control mAb, and expression of CD81 was analysed by flow cytometry. In the histograms, the grey shaded graph represents the background staining of PBLs with the isotype control mAb, the red line represents staining of PBLs from a CD81 homozygous KO mouse with the anti-CD81 mAb (A) and the blue line represents staining of PBLs from a WT mouse with the anti-CD81 mAb (B).

Initially, $CD81^{-/-}$ mice were bred together to generate a colony of homozygous $CD81^{-/-}$ mice. However these mice produced few offspring, so a $CD81^{-/-}$ colony could not be established. Therefore, $CD81^{+/-}$ mice were always bred together, and their offspring were screened to identify homozygous $CD81^{-/-}$ mice that were positive for the neomycin resistance gene and negative for the expression of CD81 on the surface of PBL. These animals, together with $CD81^{+/+}$ controls (negative for the neomycin resistance gene and positive for the expression of CD81), were used for experiments. $CD81^{+/-}$ mice (positive for the neomycin resistance gene and positive for the expression of CD81) were used as future breeding stock.

5.2.6. Characterisation of spleen DCs from CD81 KO mice

The DC subsets present in $CD81^{-/-}$ mice were not characterised in prior studies of these animals. I thus investigated the effect of CD81 deletion on the number and subset composition of DCs in the spleen. $CD81^{-/-}$ mice had spleens of normal size, and the total number of splenocytes recovered from $CD81^{-/-}$ spleens was also normal (data not shown). The proportion of $CD11c^{+}$ cells in the spleen and the MFI of CD11c expression on $CD11c^{+}$ cells from $CD81^{-/-}$ mice were also indistinguishable from those in $CD81^{+/+}$ control mice (Fig. 5.11-A). 26 % of $CD11c^{+}$ cells (DCs) from $CD81^{-/-}$ spleens were $CD4^{+}$ DCs, while 16 % of $CD11c^{+}$ cells were $CD8^{+}$ DCs (Fig. 5.11-B). 21 % of $CD11c^{+}$ cells from $CD81^{-/-}$ spleens were $B220^{+}$ plasmacytoid DCs (Fig. 5.11-C). The proportion of spleen DCs of each subset was indistinguishable from that in $CD81^{+/+}$ mice, and consistent with observations made in previous studies (Montoya *et al.*, 2005).

Further experiments addressed the expression of MHC and co-stimulatory molecules on spleen DC subsets from $CD81^{-/-}$ mice. As expected, $CD4^{+}$ and $CD8^{+}$ DCs from WT

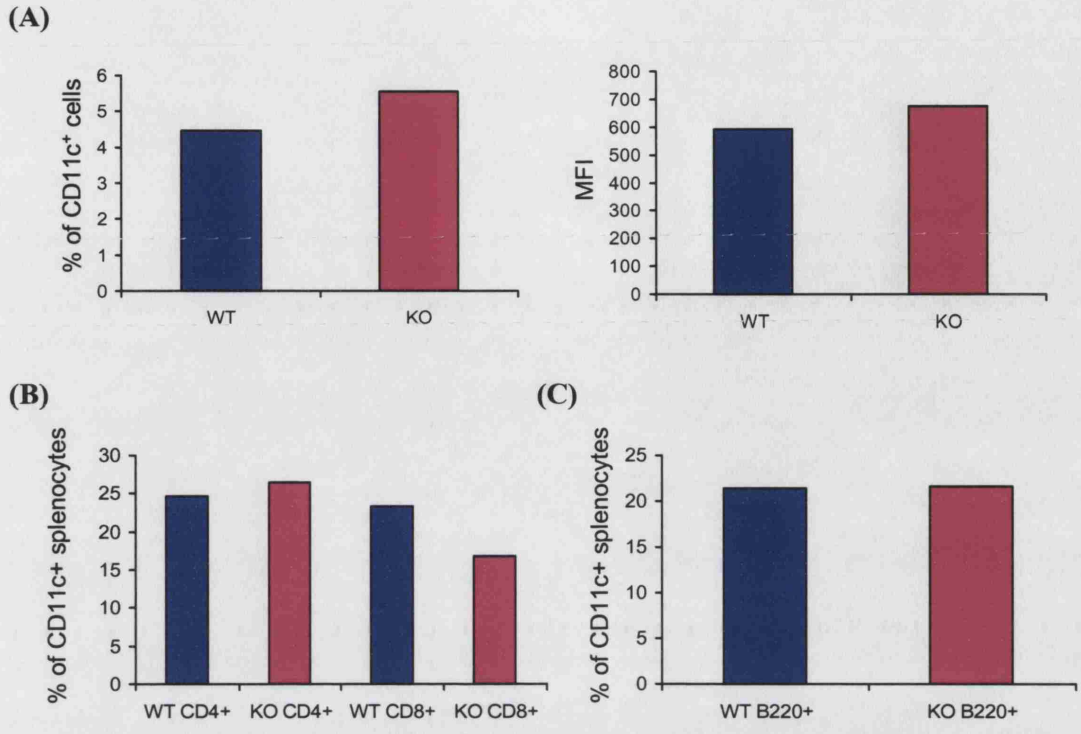


Figure 5.11. Characterisation of CD11c$^+$ cells from the spleens of CD81 KO mice.

Splenocytes were isolated as described in the Materials and Methods, and the % of cells expressing CD11c was determined by staining with an anti-CD11c mAb. The % of CD11c$^+$ cells in the total splenocyte population and the mean fluorescence intensity (MFI) of CD11c-specific staining of cells from wild-type mice (WT; blue bars) and CD81 KO mice (KO; pink bars) are shown in (A). CD11c$^+$ DCs were then purified from total splenocytes as described in the Materials and Methods. The % of CD4$^+$ and CD8$^+$ DCs in the CD11c$^+$ populations purified from WT and CD81 KO mice were determined by staining with mAbs to CD4, CD8 and CD11c (B); and the % of plasmacytoid DCs (B220$^+$ DX5$^-$) was determined by staining with mAbs to B220 and DX5 (C). The results shown are representative of findings made in 2 independent experiments.

mice expressed moderate levels of MHC class I and II molecules, and a low level of CD54 (ICAM-1), but expression of the co-stimulatory molecules CD40, CD80 and CD86 was extremely low, scarcely greater than the level of staining with isotype-matched control mAbs (Fig. 5.12). The phenotype of $CD4^+$ and $CD8^+$ spleen DCs from $CD81^{-/-}$ mice was not appreciably different from that of their counterparts from $CD81^{+/+}$ mice. Although the MFI of staining of conventional DCs from $CD81^{-/-}$ mice with mAbs to CD40 and CD80 appeared marginally higher than that of DCs from $CD81^{+/+}$ mice in the experiment depicted in Fig. 5.12, an observation also reproduced in a second experiment, the validity of this difference is unclear, as in both sets of mice, the co-stimulatory molecule staining was scarcely above background.

Plasmacytoid DCs express lower levels of MHC and co-stimulatory molecules than conventional DCs (Fig. 5.13). A low level of expression of MHC class I and class II molecules was detected on $B220^+$ DCs from WT mice, but there was very little specific staining with mAbs to $CD54^+$ and the co-stimulatory molecules CD40, CD80 and CD86 (Fig. 5.13). There was no difference in the phenotype of plasmacytoid DCs from $CD81^{+/+}$ and $CD81^{-/-}$ mice (Fig. 5.13).

5.2.7. Stimulation of bone marrow-derived DCs from CD81 KO mice with LPS, poly(I:C) and an anti-CD40 mAb

Bone marrow-derived DCs (BMDCs) were generated from $CD81^{+/+}$ and $CD81^{-/-}$ mice by *in vitro* culture of bone marrow cells with GM-CSF. Expression of CD11c, which is up-regulated as bone marrow cells differentiate into DCs in culture, was monitored over time. During the differentiation process, the production of cells expressing CD11c increased at an equivalent rate in $CD81^{+/+}$ and $CD81^{-/-}$ cell cultures, indicating that there was no defect in the ability of $CD81^{-/-}$ bone marrow cells to differentiate into DCs (Fig.

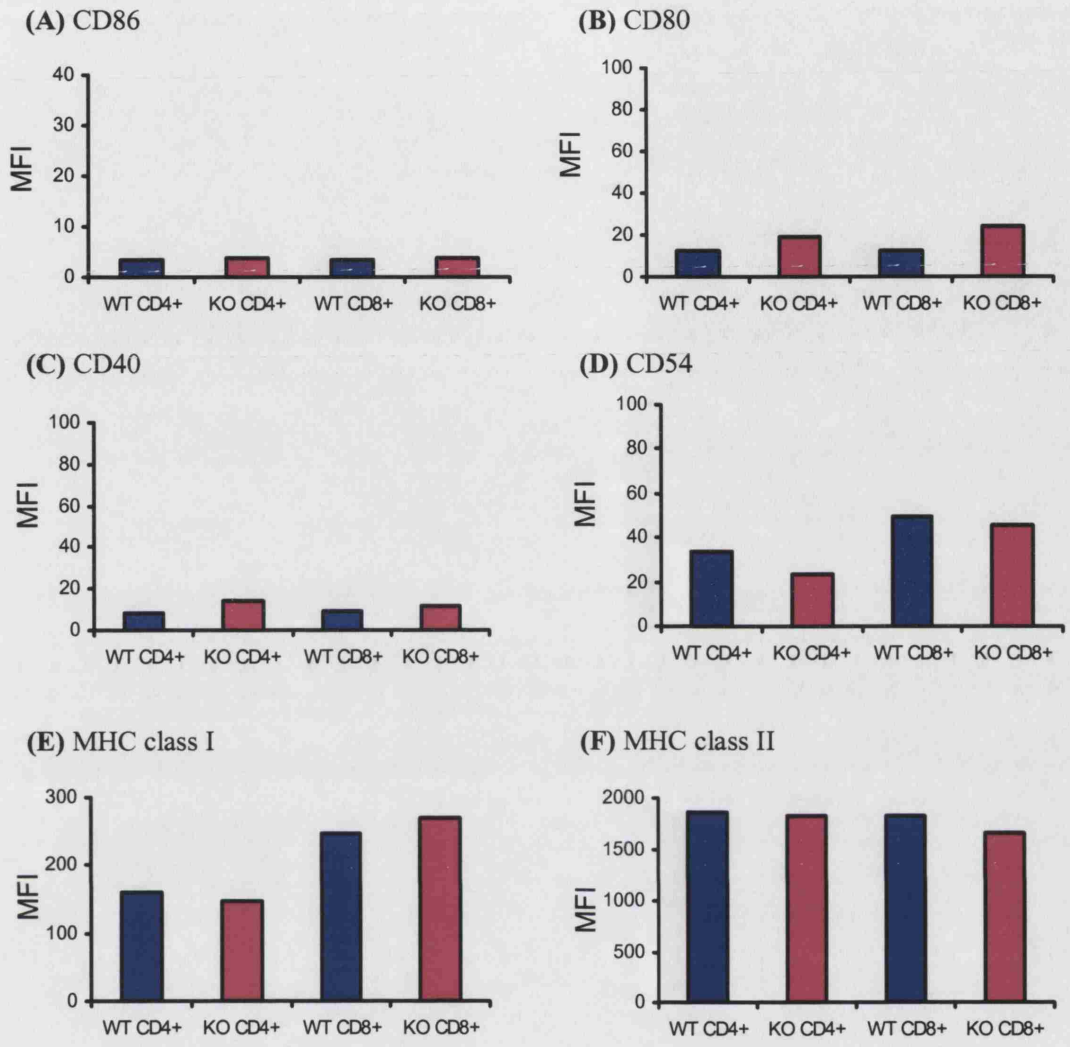


Figure 5.12. Analysis of the phenotype of $CD4^+$ and $CD8^+$ spleen DCs from CD81 KO and WT mice.

Spleen $CD11c^+$ DCs were isolated as described in the Materials and Methods. DCs were co-stained with anti-CD4 or anti-CD8 mAbs together with mAbs against different cell surface markers: CD86 (A), CD80 (B), CD40 (C), CD54 (D), MHC class I (E) and MHC class II (F). The staining was analysed by flow cytometry, gating on $CD4^+$ or $CD8^+$ cells and analysing the mean fluorescence intensity (MFI) of staining of gated cells with mAbs against the indicated surface markers. The blue bars show results from wild-type (WT) mice and the pink bars show results from CD81 KO (KO) mice. The data shown are representative of findings made in 2 independent experiments.

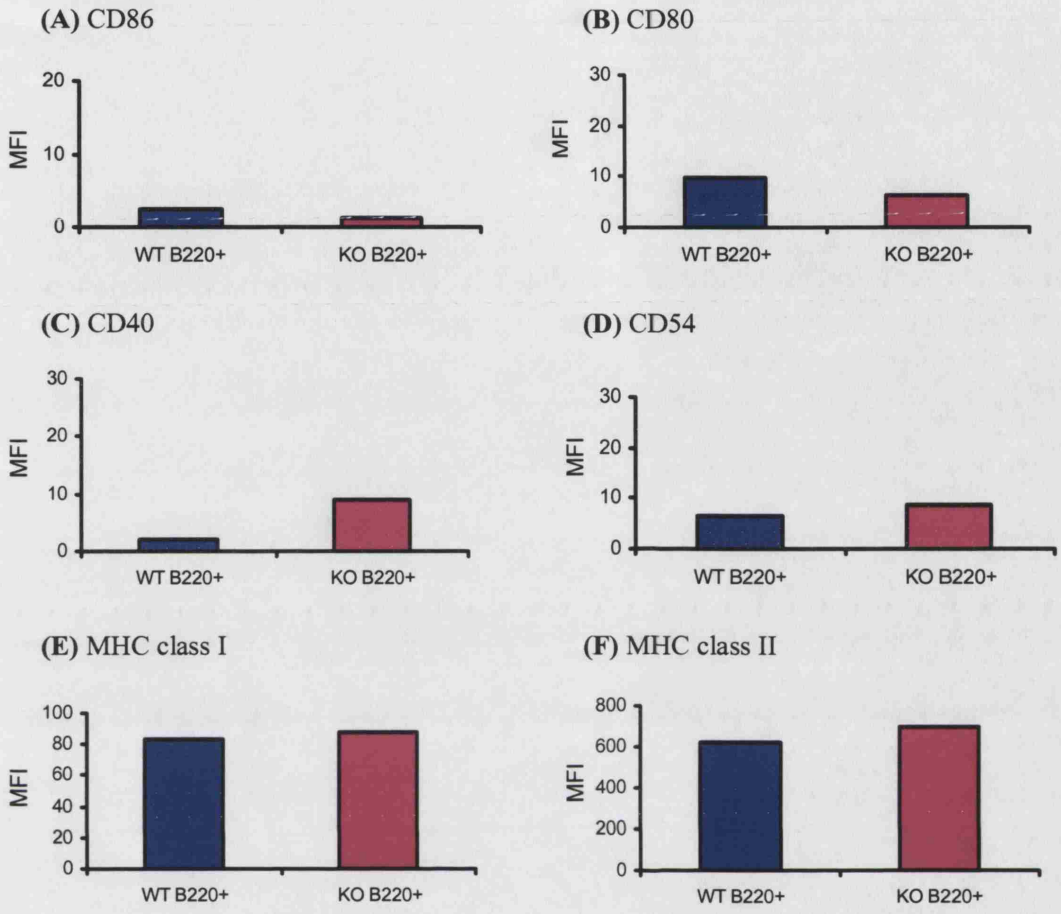


Figure 5.13. Analysis of the phenotype of B220$^+$ DX5$^-$ plasmacytoid DCs from CD81 KO and WT mice.

Spleen CD11c$^+$ DCs were isolated as described in the Materials and Methods. DCs were co-stained with anti-B220 and anti-DX5 mAbs together with mAbs against different cell surface markers: CD86 (A), CD80 (B), CD40 (C), CD54 (D), MHC class I (E) and MHC class II (F). The staining was analysed by flow cytometry, gating on B220$^+$DX5$^-$ cells and analysing the mean fluorescence intensity (MFI) of staining of gated cells with mAbs against the indicated surface markers. The blue bars represents results from wild-type (WT) mice and the pink bars represent results from CD81 KO (KO) mice. The data shown are representative of findings made in 2 independent experiments.

5.14-A). Interestingly, $CD81^{-/-}$ BMDCs were found to express higher levels of CD11c than $CD81^{+/+}$ BMDCs on day 8 (Fig. 5.14-B). However, BMDCs derived from $CD81^{+/+}$ and $CD81^{-/-}$ mice were otherwise almost indistinguishable phenotypically, expressing very similar levels of MHC class I and II, CD54 and co-stimulatory molecules (see untreated BMDC data in Fig. 5.15, 5.17 and 5.19). The one difference between them was that CD81 expression was detected on 52 % of $CD81^{+/+}$ BMDCs, whereas $CD81^{-/-}$ BMDCs did not express this molecule (data not shown).

The response of $CD81^{-/-}$ BMDCs to TLR4, TLR3 and CD40 stimulation was then investigated. BMDCs generated from $CD81^{+/+}$ and $CD81^{-/-}$ mice were found to be activated by LPS in a dose-dependent manner, up-regulating co-stimulatory molecules, CD54, MHC class I and to a lesser extent MHC class II (Fig. 5.15). There was little difference in the phenotypic activation of BMDCs from $CD81^{+/+}$ and $CD81^{-/-}$ mice, although the increase in the MFI of CD86 staining on $CD81^{-/-}$ BMDCs was slightly greater than that on $CD81^{+/+}$ BMDCs (Fig. 5.15-B). This was observed in 2 independent experiments carried out. LPS-stimulated cytokine production was also analysed. Both $CD81^{+/+}$ and $CD81^{-/-}$ BMDCs were found to produce IL-10, TNF-α, MCP-1, IL-6 and IL-12 p70 in response to LPS stimulation in a dose-dependent manner (Fig. 5.16). No difference was observed in cytokine production by BMDCs from WT and CD81 KO mice.

Poly(I:C) also activated BMDCs from $CD81^{+/+}$ and $CD81^{-/-}$ mice in a dose-dependent manner, as evidenced by an increase in surface expression of MHC class I, CD54 and co-stimulatory molecules (Fig. 5.17). In 2 independent experiments, there was little difference in poly(I:C) stimulated phenotypic activation of BMDCs from $CD81^{+/+}$ and

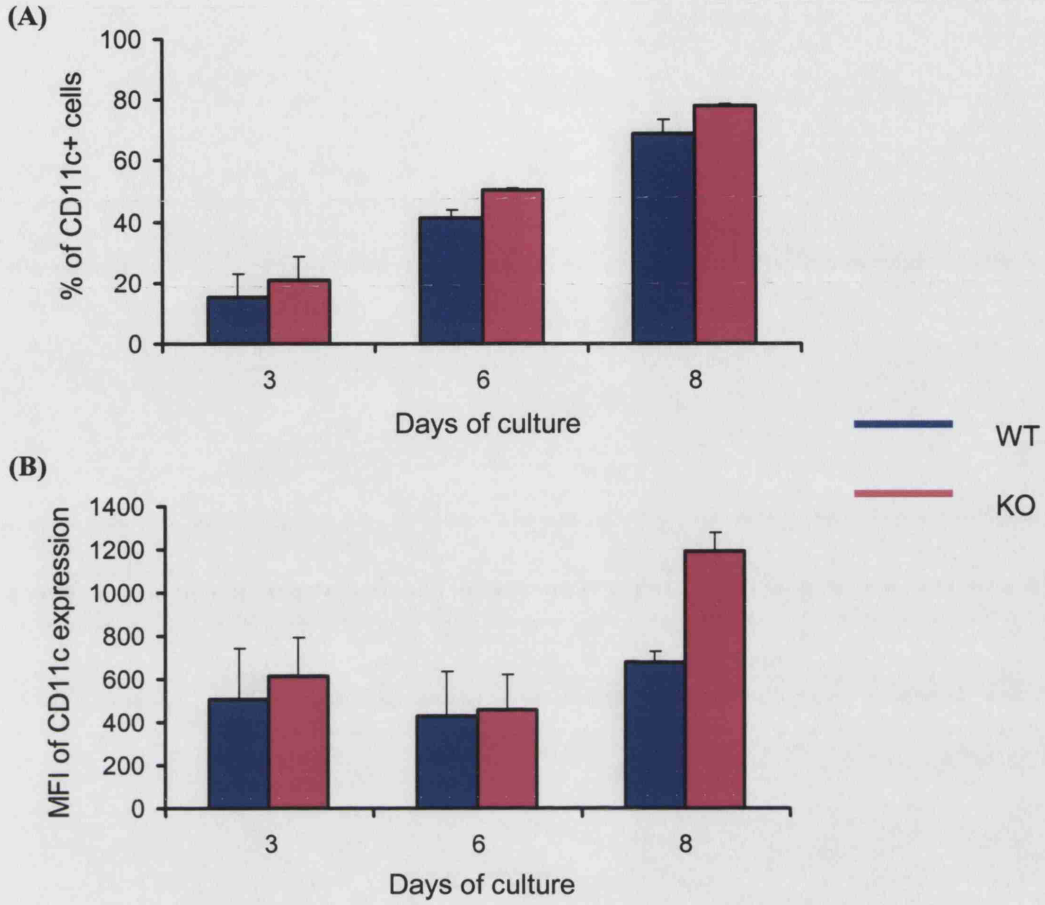


Figure 5.14. Analysis of the differentiation of bone marrow cells from WT and CD81 KO mice into DCs during *in vitro* culture.

Bone marrow (BM) was extracted from wild-type (WT) and CD81 KO mice (KO) as described in the Materials and Methods. BM cells were cultured *in vitro* for 8 days in GM-CSF-containing medium, and their differentiation into DCs was assessed by monitoring surface expression of CD11c on days 3, 7, and 8 of culture. The % of cells expressing CD11c is shown in (A), and the mean fluorescence intensity (MFI) of CD11c staining on $CD11c^+$ cells is shown in (B). The blue bars show results from WT mice and the pink bars show results from CD81 KO mice. The data shown are the mean of results obtained in 2 independent experiments; the error bars indicate one standard error above the mean.

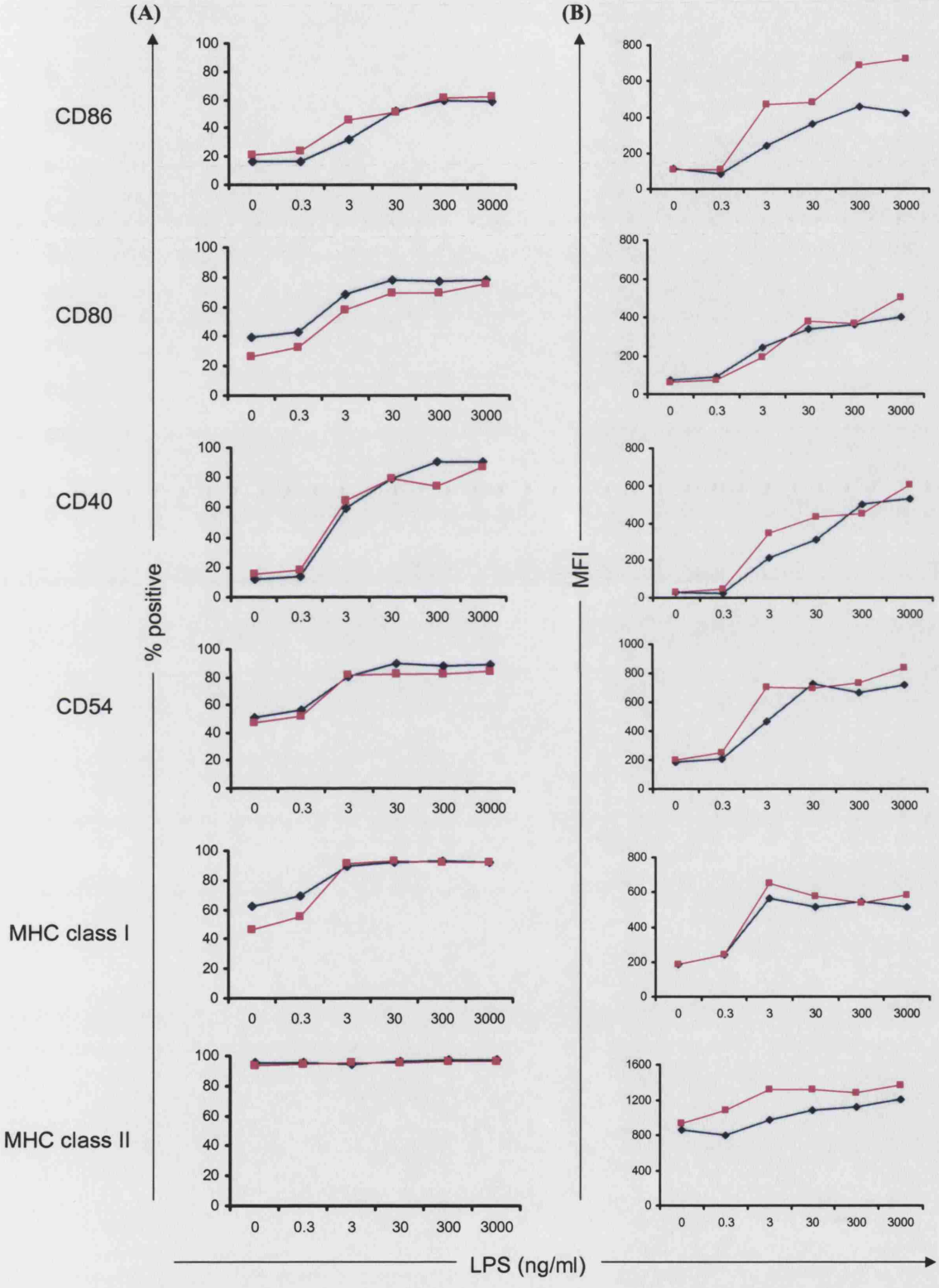

(A)
(B)
CD86
CD80
CD40
CD54
MHC class I
MHC class II
% positive
MFI
LPS (ng/ml)
0
0.3
3
30
300
3000

Figure 5.15. Analysis of the phenotypic maturation of BMDCs from WT or CD81 KO mice in response to LPS stimulation.

Bone marrow-derived DCs (BMDCs) generated from wild-type (WT) and CD81 KO (KO) mice were stimulated with different concentrations of LPS (0-3000 ng/ml) overnight, then were stained with mAbs against different surface markers (CD86, CD80, CD40, CD54, MHC class I, MHC class II) and analysed by flow cytometry. The % of BMDCs expressing the indicated surface markers is shown in (A). The mean fluorescence intensity (MFI) of staining of all cells is shown in (B). The blue lines represent BMDCs from WT mice and the pink lines represent BMDCs from CD81 KO mice. The results shown are representative of findings made in 2 independent experiments.

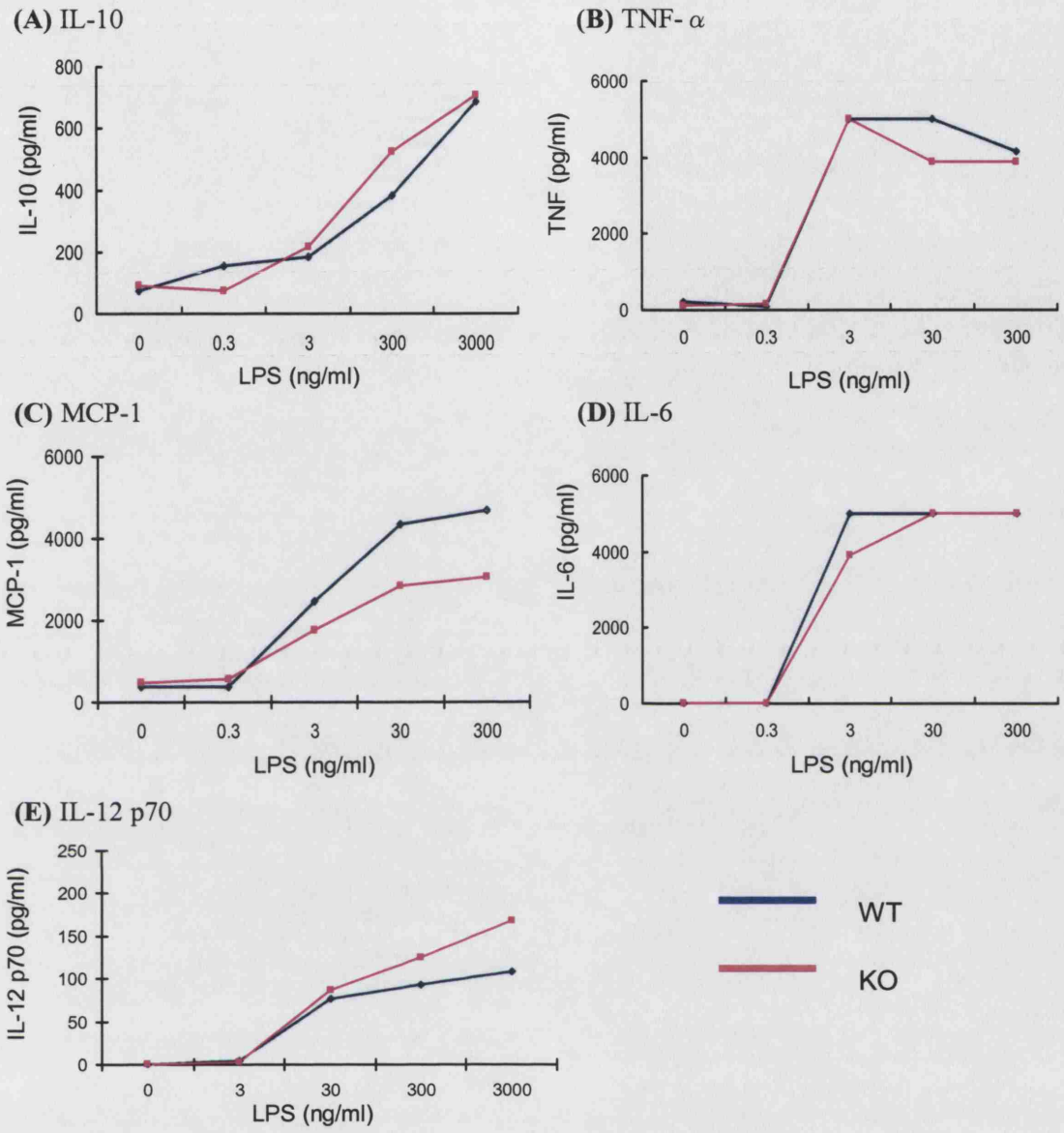


Figure 5.16. Analysis of cytokine production by BMDCs from WT and CD81 KO mice in response to LPS stimulation.

Bone marrow-derived DCs (BMDCs) were stimulated with different concentrations of LPS (0-3000 ng/ml) overnight, then supernatants were harvested. Levels of IL-10 (A), TNF-α (B), MCP-1 (C) and IL-6 (D) were determined by CBA assay. Levels of IL-12 p70 (E) were measured by ELISA. The blue lines represent cytokine levels in BMDC supernatants from wild-type (WT) mice and the pink lines represent cytokine levels in BMDC supernatants from CD81 KO (KO) mice. The results shown are representative of findings made in 2 independent experiments.

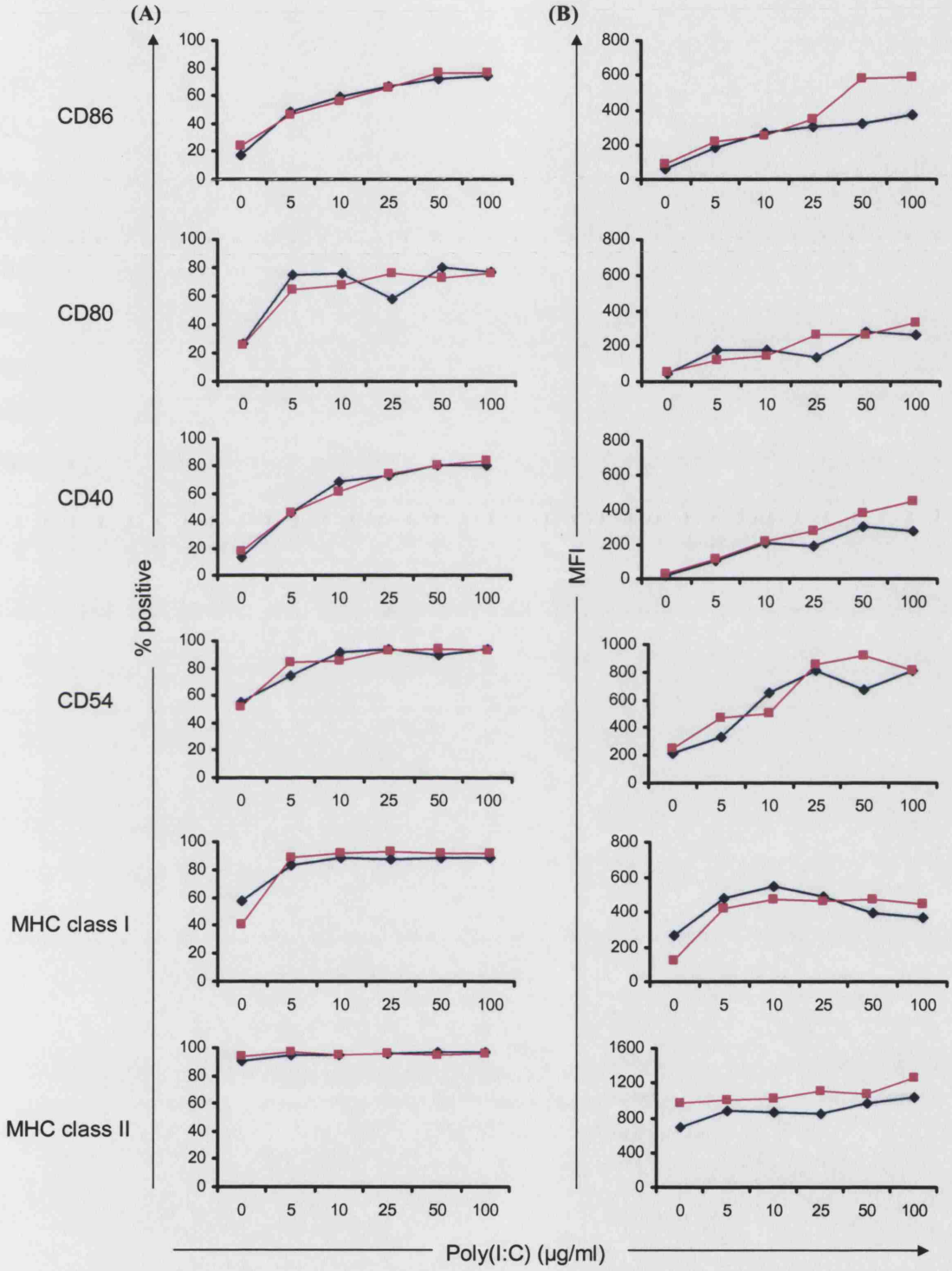

(A)
(B)
CD86
CD80
CD40
CD54
MHC class I
MHC class II
% positive
MFI
Poly(I:C) (μg/ml)
0
5
10
25
50
100

Figure 5.17. Analysis of the phenotypic maturation of BMDCs from WT and CD81 KO mice in response to poly(I:C).

Bone marrow-derived DCs (BMDCs) generated from wild-type (WT) and CD81 KO (KO) mice were stimulated with different concentrations of poly(I:C) (0-100 μg/ml) overnight, then were stained with mAbs against different surface markers (CD86, CD80, CD40, CD54, MHC class I, MHC class II) and analysed by flow cytometry. The % of BMDCs expressing the indicated surface markers is shown in (A). The mean fluorescence intensity (MFI) of staining on all cells is shown in (B). The blue lines represent BMDCs from WT mice and the pink lines represent BMDCs from CD81 KO mice. The results shown are representative of findings made in 2 independent experiments.

$CD81^{-/-}$ mice. BMDCs were also stimulated to produce cytokines (IL-10, TNF-α, MCP-1, IL-6 and IL-12) in response to poly(I:C) in a dose-dependent manner (Fig. 5.18). There was no difference observed in cytokine production by BMDCs from WT and CD81 KO mice.

Anti-CD40 mAb cross-linking was the weakest stimulus of all, inducing only a slight increase in expression of co-stimulatory molecules on BMDCs from both $CD81^{+/+}$ and $CD81^{-/-}$ mice (Fig. 5.19). A dose-dependent increase in production of both IL-6 and IL-12 p70 was also stimulated by the anti-CD40 mAb (Fig. 5.20), but again, in 2 experiments, no consistent differences were observed in the response of BMDCs from WT and CD81 KO mice. Although IL-12 p70 production by $CD81^{-/-}$ BMDCs was lower than that of $CD81^{+/+}$ BMDCs in the experiment depicted in Fig. 5.20, this difference was not observed in a second experiment.

In conclusion, BMDCs from $CD81^{-/-}$ mice were responsive to LPS, poly(I:C) and anti-CD40 stimulation, with no major qualitative or quantitative differences being observed between the responses of BMDCs from $CD81^{+/+}$ and $CD81^{-/-}$ mice to these stimuli.

5.3. Discussion

CD81 is expressed on both conventional and plasmacytoid DCs in humans, though the latter express very low levels of this molecule. Additionally, monocyte-derived DCs also express CD81. In parallel, mouse DCs have been reported to express CD81 (Maecker *et al.*, 2000). Despite the expression of CD81 on DCs, the function(s) of CD81 on these cells are not known. In this chapter, I addressed the hypothesis that

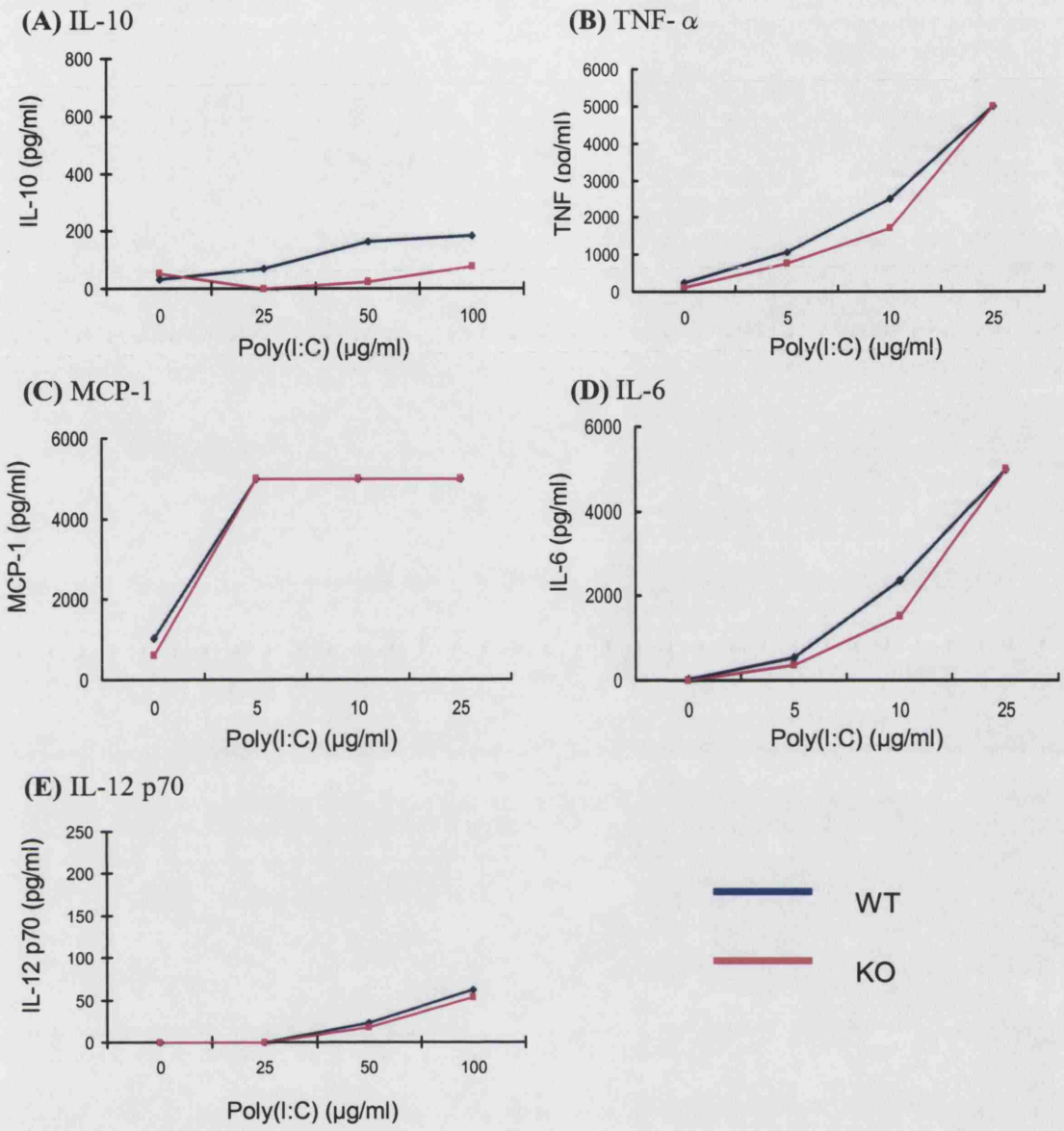


Figure 5.18. Analysis of cytokine production by BMDCs from WT and CD81 KO mice in response to poly(I:C) stimulation.

Bone marrow-derived DCs (BMDCs) were stimulated with different concentrations of poly(I:C) (0-100 µg/ml) overnight, then supernatants were harvested. Levels of IL-10 (A), TNF-α (B), MCP-1 (C) and IL-6 (D) were determined by CBA assay. Levels of IL-12 p70 (E) were measured by ELISA. The blue lines represent cytokine levels in BMDC supernatants from wild-type (WT) mice and the pink lines represent cytokine levels in BMDC supernatants from CD81 KO (KO) mice. The results shown are representative of findings made in 2 independent experiments.

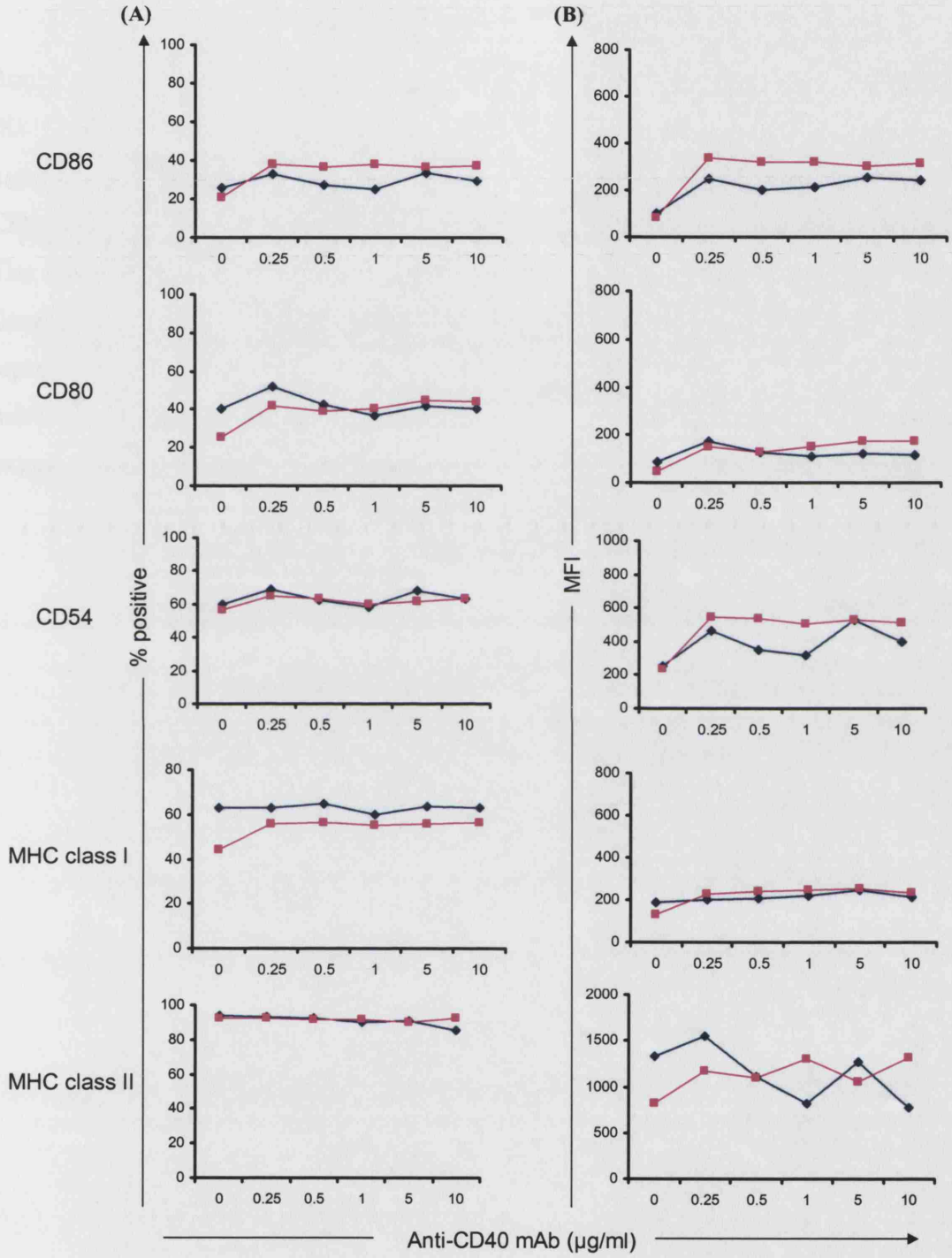

(A)
(B)
CD86
CD80
CD54
MHC class I
MHC class II
% positive
MFI
0
0.25
0.5
1
5
10
Anti-CD40 mAb (μg/ml)

Figure 5.19. Analysis of the phenotypic maturation of BMDCs from WT or CD81 KO mice in response to stimulation with an anti-CD40 mAb.

Bone marrow-derived DCs (BMDCs) generated from wild-type (WT) and CD81 KO (KO) mice were stimulated with different concentrations of anti-CD40 mAb (0-10 μg/ml) overnight, then were stained with mAbs against different surface markers (CD86, CD80, CD40, CD54, MHC class I, MHC class II) and analysed by flow cytometry. The % of BMDCs expressing the indicated surface markers is shown in (A). The mean fluorescence intensity (MFI) of staining of all cells is shown in (B). The blue lines represent BMDCs from WT mice and the pink lines represent BMDCs from CD81 KO mice. The results shown are representative of findings made in 2 independent experiments.

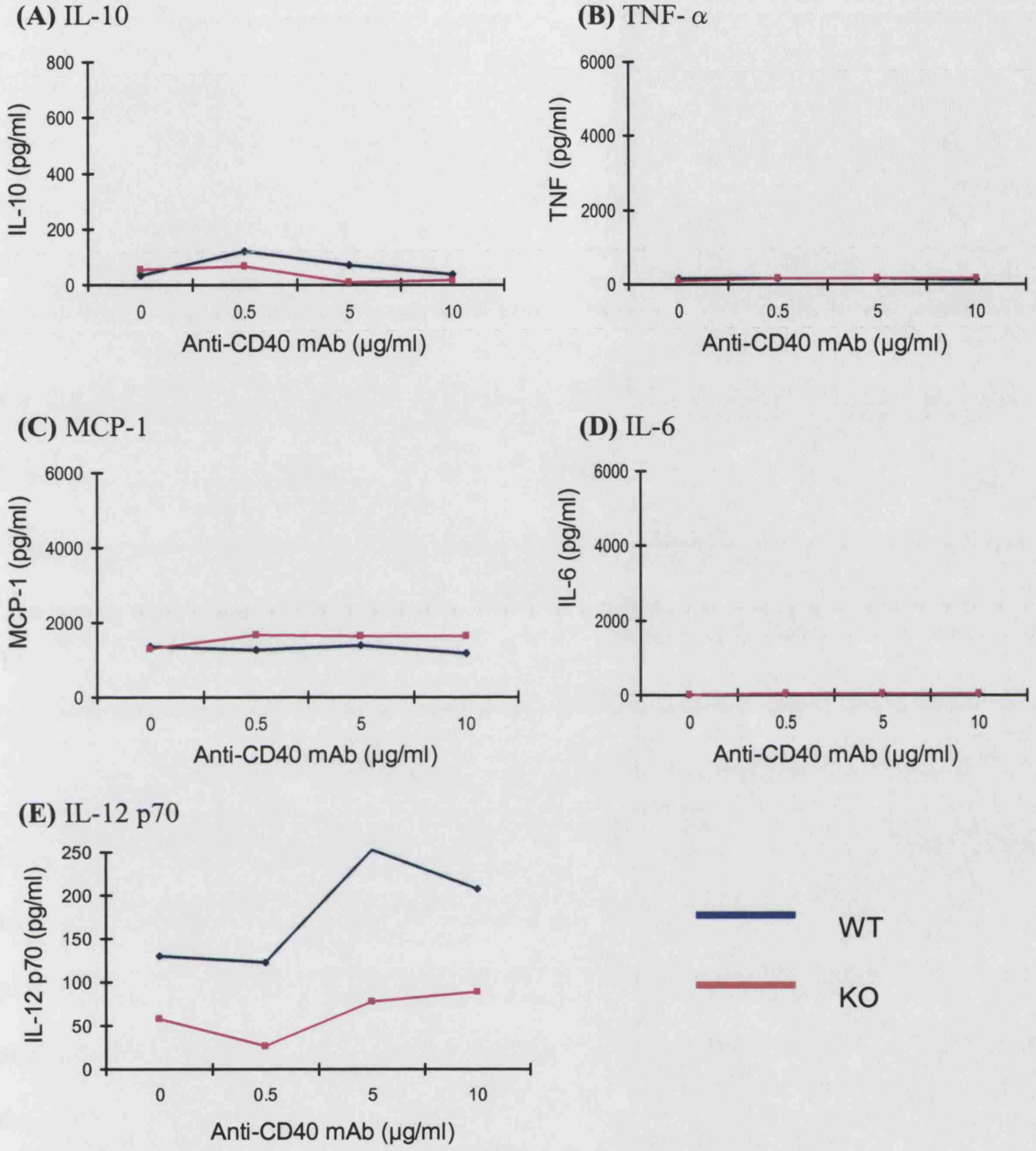


Figure 5.20. Analysis of cytokine production by BMDCs from WT and CD81 KO mice in response to stimulation with anti-CD40 mAb.

Bone marrow-derived DCs (BMDCs) were stimulated with different concentrations of anti-CD40 mAb (0-10 µg/ml) overnight, then supernatants were harvested. Levels of IL-10 (A), TNF-α (B), MCP-1 (C), IL-6 (D) and IL-12 p70 (E) were determined by CBA assay. The blue lines represent cytokine levels in BMDC supernatants from wild-type (WT) mice and the pink lines represent cytokine levels in BMDC supernatants from CD81 KO (KO) mice. The results shown are representative of findings made in 2 independent experiments.

CD81 may modulate the response of DCs to activating stimuli, analogous to its role on T and B cells.

Human T and B cells are reported to down-regulate CD81 expression on their surface after activation (Fritzsching *et al.*, 2002); likewise, I found that CD81 expression on monocyte-derived DCs was also down-regulated in response to activation of these cells via several different stimuli (LPS, poly(I:C) and an anti-CD40 mAb). These results suggest that the regulation of CD81 expression in response to activating stimuli may be conserved in many cell types in humans. However, it is not clear whether CD81 forms part of the activation complex on DCs, in the same way that it is associated with the TCR and BCR complex on T and B cells, or whether it is merely a marker for cell activation.

In order to investigate the role(s) of CD81 in the response of DCs to different stimuli, two approaches were taken in this study. First, the effects of CD81 cross-linking by plate-bound mAbs on the response of human DCs to different activating stimuli was addressed. Ideally, *ex vivo* myeloid and plasmacytoid DCs should have been used in these experiments, but the studies with blood DCs are hampered by the low number of these cells present in human peripheral blood. Hence, as an alternative, *in vitro*-differentiated monocyte-derived DCs are frequently used in experimental studies and show many similarities to the myeloid DCs present in the blood, but it remains unclear whether monocytes that are recruited to tissues constitutively or in response to inflammatory stimuli do develop into DCs *in vivo*. Cross-linking of CD81 by plate-bound anti CD81 mAb was shown to modulate the activation of human T cells (chapter 4). Further, the anti-CD81 mAb used in this study was able to bind to CD81 expressed

on human monocyte-derived DCs (chapter 3 and Fig. 5.2). However, as the expression of CD81 on monocyte-derived DCs was low, it was possible that CD81 on DCs may not have been efficiently cross-linked using the plate-bound mAb system. This was a drawback to this approach.

The second approach taken was to study the response of DCs derived from $CD81^{-/-}$ mice to activating stimuli. A possible drawback to use KO mice is that cells developing in a KO environment can sometimes compensate for missing components or functions. Thus, it is plausible that the function(s) of CD81 might be substituted for in $CD81^{-/-}$ mice by other molecules such as other members of tetraspanin superfamily. Hence no abnormalities may be apparent in processes/pathways that CD81 is normally involved in. I did not see any abnormalities in the number, subset composition or phenotype of spleen DCs from $CD81^{-/-}$ mice. I also observed no difference in the response (as assessed by phenotypic activation and cytokine production) of bone marrow-derived DCs from CD81 KO and WT mice to activating stimuli. Although BMDCs are frequently studied as surrogates for conventional DCs, they do not reproduce all the properties of the heterogeneous populations of conventional DCs in the murine spleen. Had time permitted, the responses of different spleen DC sub-populations from $CD81^{-/-}$ mice would have been investigated.

It was hypothesised based on the role of CD81 on T cells that CD81 on DCs may co-ordinate the association of cell surface molecules and facilitate signalling triggered by the receptor complex(es) with which CD81 is closely associated. CD81 is known to cluster with CD14 and TLR4 on monocytes (Pfeiffer *et al.*, 2001). Interestingly, TLR4-MD2 is not present in lipid rafts on monocytes but is recruited around raft-resident CD14 after LPS stimulation (Miyake, 2004; Triantafilou *et al.*, 2002). In addition, the

association of CD81 with CD14 is only seen after LPS stimulation (Pfeiffer *et al.*, 2001). In addition to monocytes, TLR4 is expressed on myeloid DCs, human monocyte-derived DCs and mouse BMDCs (Reis e Sousa, 2004), and all are activated by the TLR4 ligand LPS, which is a principal component of the outer membrane of Gram negative bacteria, via both MyD88-dependent and -independent signalling pathways. Thus I investigated whether response of DCs to LPS was affected by CD81 cross-linking or by the absence of CD81. However, CD81 cross-linking did not alter the response of monocyte-derived DCs to LPS. In addition, BMDCs from $CD81^{-/-}$ mice responded to LPS normally. I thus did not obtain any evidence from either system that CD81 modulates signalling through TLR4. It is possible that CD81 may not be associated with TLR4 on monocyte-derived DCs. Had time permitted, this is something that would have been of interest to address. Alternatively, it is plausible that CD81 has no role in the facilitation of the formation of efficient TLR4 receptor complexes on monocyte-derived DCs. Supporting this, there is as yet no evidence to demonstrate the functional importance of all the molecules recruited to CD14 molecular complexes on monocytes in LPS-inducing signalling (Underhill, 2003).

I also addressed the role of CD81 in modulating signalling through TLR3, because signalling differs from that via TLR4, being completely MyD88 independent. Like TLR4, TLR3 is also expressed on conventional DCs, human monocyte-derived DCs and mouse BMDCs (Reis e Sousa, 2004), and all can be activated by the TLR3 ligand poly(I:C) (Alexopoulou *et al.*, 2001). Poly(I:C) is a synthetic analogue of dsRNA, which is generated during the replication of many viruses. I investigated whether the response of DCs to poly(I:C) was affected by CD81 cross-linking or by the absence of CD81. CD81 cross-linking had no effect on the activation of monocyte-derived DCs in

response to TLR3 stimulation, and BMDCs from $CD81^{-/-}$ mice responded to poly(I:C) as well as those from WT mice. Again, I could not find any evidence from either system that CD81 modulates signalling through TLR3. In contrast to fibroblasts, where TLR3 is expressed on the cell surface, TLR3 is predominantly expressed in endosomal compartments in monocyte-derived DCs and internalised poly(I:C) activates TLR3 to transduce signals inside the cells (Matsumoto *et al.*, 2003). CD81 itself does not carry signalling motifs, so it needs to associate with signalling receptor complexes to mediate immunomodulatory effects. Thus it is not surprising that CD81 cross-linking did not affect the TLR3-signalling pathway in monocyte-derived DCs.

Another mechanism by which DCs can be activated is via ligation of CD40. Again, CD40 is expressed on conventional DCs, human monocyte-derived DCs and mouse BMDCs (MacDonald *et al.*, 2002; O'Sullivan & Thomas, 2003). The natural ligand of CD40 is CD154, which is expressed as a homotrimer on activated $CD4^+$ T cells (Hermans *et al.*, 1999; Schoenberger *et al.*, 1998; Shreedhar *et al.*, 1999). Ligation of CD40 can induce the maturation of DCs and their migration into secondary lymphoid tissues, and provides a major signal for secretion of IL-12 by DCs, effecting Th1 T-cell differentiation. It has also been shown that DCs matured by CD40 ligation by $CD4^+$ T cells, unlike DCs matured by LPS, are capable of fully activating naïve $CD8^+$ T cells, leading to generation of effector functions and memory cell (Kelleher & Beverley, 2001; Schuurhuis *et al.*, 2000; Sun & Bevan, 2003). In this study, an anti-CD40 mAb was used to mimic stimulation with membrane-bound CD40 ligand, and then I investigated whether response of DCs to CD40 ligation was affected by CD81 cross-linking or by the absence of CD81. The anti-CD40 mAb was the weakest inducer of DC maturation of the stimuli used in this study. Both human monocyte-derived DCs and

BMDCs did not show full phenotypic activation after stimulation with the anti-CD40 mAb. Moreover, although CD40 ligation induced production of IL-12 by DCs, other pro-inflammatory cytokines were not produced at high levels (Fig. 5.8). In many other studies, CD40 ligation was found to induce greater phenotypic activation of DCs (both monocyte-derived DCs and mouse BMDCs) (Nakamura *et al.*, 2004; Terheyden *et al.*, 2000; Watanabe *et al.*, 2003), and to elicit the production of inflammatory cytokines such as IL-6 and TNF-α as well as IL-10 (Megiovanni *et al.*, 2004) in addition to IL-12. It has been reported that soluble purified CD40 ligand and CD40 ligand expressed on cell lines had a superior ability to induce the expression of co-stimulatory molecules on DCs compared to soluble agonistic anti-CD40 mAbs or cross-linking of CD40 by mAbs (Cognasse *et al.*, 2005). Moreover, Nakamura *et al.* reported that optimal maturation of mouse BMDCs by anti-CD40 mAb was achieved after a 48-hour incubation (Nakamura *et al.*, 2004), whereas I only stimulated my cells overnight. Thus it is likely that the conditions used in this study for the activation of DCs by CD40 ligation were sub-optimal. Had time allowed, alternative means if achieving CD40 ligation and longer incubation periods could have been used to achieve full maturation of DCs through CD40, and the role of CD81 in this process investigated. However in the experiments I carried out using either monocyte-derived DCs or BMDCs, I found no evidence that CD81 modulates DC maturation/activation through CD40 signalling.

Miyazaki *et al.* reported that B cell proliferation in response to anti-CD40 mAb, LPS and anti-IgM mAb was altered in $CD81^{-/-}$ mice (Miyazaki *et al.*, 1997), raising the possibility that the deletion of CD81 might also affect the response of DCs to LPS and anti-CD40 mAbs. However, my results suggest that CD81 is not involved in modulating (either quantitatively or qualitatively) the response of DCs to stimulation through TLR4,

TLR3 or CD40. The function of CD81 on DCs may thus be different from that on T and B cells, where CD81 associates with the major immunoreceptor complexes to modulate signalling. Alternatively, it is plausible that CD81 is associated with surface molecules on DCs that were not investigated in this study, and modulates signalling through those receptor complexes. It is also possible that CD81 cross-linking might have had effects on DC functions which were not examined in this study, for example modulating the expression of different surface markers, production of other cytokines and chemokines and/or DC interaction with T cells.

I also found in this study that CD81 is not required for the development of DCs in mice. $CD81^{-/-}$ mice had normal numbers and a normal subset composition of spleen DCs. Further, bone marrow cells from CD81 KO mice developed into BMDCs *in vitro* normally. Although $CD81^{-/-}$ BMDCs were found to express higher levels of CD11c than $CD81^{+/+}$ BMDCs (Fig. 5.14), this was not observed on *ex vivo* spleen DCs (Fig. 5.11). It is not clear whether the enhanced expression of CD11c on $CD81^{-/-}$ BMDCs has any significance. CD11c is a member of the integrin family which is involved in cell-cell contact and cell-extracellular matrix interaction. It is plausible that CD11c was up-regulated to compensate the deficiency of CD81 in KO mice, since CD81 is also involved in cell adhesion (Levy *et al.*, 1998). Thus had time permitted, it would have been interesting to explore whether deficiency of CD81 affects the localisation of DCs within spleen and non-lymphoid tissues and/or DC migration following *in vivo* activation by infectious stimuli.

A major role of conventional DCs is to mediate antigen presentation to T cells. It is plausible that CD81 may be involved in the antigen presentation process. It has been

reported that CD81 as well as other members of the tetraspanin superfamily, CD9 and CD53, are clustered around MHC class II molecules on the surface of immature human DCs (Engering & Pieters, 2001). It is also reported that members of the tetraspanin superfamily including CD81 are enriched in CDw78-MHC class II microdomains, where MHC class II molecules presenting selected antigenic peptides are brought together to mediate optimal activation of antigen-specific $CD4^+$ T cells. On DCs, CDw78-tetraspanin microdomains appear in non-lipid rafts (Vogt *et al.*, 2002), although after stimulation, MHC class II-peptide complexes are also recruited to classical lipid rafts (Meyer zum Bueschenfelde *et al.*, 2004). Interestingly, tetraspanin family members are thought to contribute to the formation of CDw78 microdomains (Vogt *et al.*, 2002); CD81 may be among the proteins involved in this process. Another possible role of tetraspanin proteins in activating T cells is in the formation of microvesicles called exosomes. Exosomes are vesicles secreted from cell types including leukocytes and endothelial cells in an exocytic manner (Raposo *et al.*, 1996). They contain peptide-loaded MHC molecules, and constitute a means by which antigen can be transferred to DCs for presentation to T cells. Exosomes are known to be enriched in tetraspanin proteins including CD81, CD82 and CD63 (Escola *et al.*, 1998); and it is thought that tetraspanins including CD81 play a role in targeting exosomes to DCs for uptake (Morelli *et al.*, 2004). Hence CD81 may potentially play role in the organisation of MHC class II molecules carrying particular peptides into microdomains on APCs and/or the formation and/or transfer of MHC class II-loaded exosomes, in both cases promoting $CD4^+$ T cell priming. This possibility could have been tested using DCs and exosomes from CD81 KO mice. Antigen presentation by DCs from CD81 KO mice has not been investigated in any depth in previous studies.

No abnormalities have been reported in T cell development in $CD81^{-/-}$ mice (Maecker & Levy, 1997; Miyazaki *et al.*, 1997; Tsitsikov *et al.*, 1997), indicating that there is not a major defect in thymic selection by DCs in these animals. Further, antigen-specific $CD4^+$ T cell responses can be induced in $CD81^{-/-}$ mice (Deng *et al.*, 2000). Th2 cytokine production by T cells primed by immunisation with an antigen plus alum was found to be reduced in $CD81^{-/-}$ mice (Deng *et al.*, 2000); however this was attributed to a defect in T-B cell interaction rather than in DC functions (Deng *et al.*, 2000). Recent studies also indicate that there are no abnormalities in the virus-specific $CD8^+$ T cell response induced in $CD81^{-/-}$ mice following infection with LCMV (Burke *et al.*, unpublished observations). In summary, there does not appear to be a major deficiency in DC functions in $CD81^{-/-}$ mice, although further studies are required to elucidate whether there are subtle abnormalities in their antigen presentation properties and/or the nature of the T cell response they elicit.

Neither the molecular partners of CD81 nor functions of CD81 on plasmacytoid DCs are known. I did not have time to study the functions of CD81 on plasmacytoid DCs, but it would have been of interest to do so. Results I obtained in chapter 3 suggest that human plasmacytoid DCs express lower levels of CD81 than other haematopoietic cell subsets. It would have been interesting to determine whether spleen plasmacytoid DCs in mice also express a lower level of CD81 than other DC subsets. There may be differences in the expression and function(s) of CD81 in humans and in mice. CD81 is expressed on all T cells in humans, whereas murine naïve T cells do not express CD81 (Feigelson *et al.*, 2003), and the activation induces high levels of CD81 expression on T cells (Maecker *et al.*, 2000; Witherden *et al.*, 2000).

Overall, it is not clear what CD81 does in both human and mouse DCs. More studies are required to understand the role of CD81 on DCs, and the effect of CD81 deletion in immune cells on their response to different stimuli and infections.

Chapter 6 General discussion

It has been more than a decade since the discovery of HCV. Because of the introduction of screening methods for identification of HCV-positive blood, there has been a large reduction in transfusion-related HCV infection. However, approximately 3 % of the world's population is chronically infected with this virus (Pellicano *et al.*, 2004), and these individuals face a risk of progressing to develop hepatic diseases and/or autoimmune lymphoproliferative disorders. Although advances in molecular biology and genetics have enabled tremendous progress in understanding HCV biology, there is still a lack of effective vaccines and treatments to combat this infection.

Since the liver is the primary site of HCV replication, many studies have focused on the interaction between HCV and hepatic cells. However, there are a number of studies supporting HCV infection of cells of the immune system *in vivo* (Bouffard *et al.*, 1992) (Goutagny *et al.*, 2003; Lerat *et al.*, 1998; Muller *et al.*, 1993; Zignego *et al.*, 1992). This may explain the recurrence of hepatic HCV infection after liver transplantation of patients (Botero, 2004; Dahari *et al.*, 2005). The extrahepatic disorders with which HCV infection is tightly associated may be due at least in part to infection of haematopoietic cells (Ferri & Zignego, 2000). Further, viral infection of immune system cell subsets may cause their loss or impair their functions, potentially enhancing HCV persistence. Thus the interaction between the virus and host immune system cells is of great importance to understand if this infection is to be combated. The E2 surface glycoprotein plays a critical role in the viral life-cycle, mediating the interaction between the virus and host cell surface receptor(s). Further, it has been proposed that interaction between the E2 protein and its receptors may not only be crucial for viral entry, but may also modulate the functions of the receptor-bearing cells, potentially contributing to viral persistence/pathogenesis. In this study, the expression of the HCV (co)-receptors CD81 and SR-BI was characterised on different PBMC subsets and the

binding of E2 glycoproteins to these cells was addressed. In addition, the functions of the E2-binding receptors CD81 on NK cells and DCs were explored, focusing particularly on analysis of how CD81 cross-linking may affect the response of these cells to activating stimuli.

Consistent with the evidence for HCV infection of haematopoietic cells, I found that E2 proteins can bind to PBMC subsets (chapter 3). Moreover, all soluble E2s tested displayed a similar pattern of binding to different PBMC subsets, largely paralleling the level of cellular CD81 expression. Although CD81 plays a prominent role in HCV binding to PBMC subsets, the PBMC subsets expressing the highest levels of CD81 were not those reported to be infected by HCV *in vivo*, suggesting that CD81 is not a limiting determinant of HCV tropism *in vivo*. Instead, there was a good correlation between the ability of PBMC subsets to bind E2 via both CD81-dependent and CD81-independent mechanisms and *in vivo* infection, indicating that E2-CD81 interaction is probably not sufficient to allow infection of haematopoietic cells. This observation is in agreement with other studies suggesting that CD81 is required but not sufficient for the HCV attachment/entry process (Bartosch *et al.*, 2003a; Bartosch *et al.*, 2003b; Cormier *et al.*, 2004; Hsu *et al.*, 2003; McKeating *et al.*, 2004). SR-BI was found to be expressed on most, but not all of the PBMC subsets reported to be HCV replication sites *in vivo*, suggesting that it may be involved in mediating HCV infection of haematopoietic cells. However, additional receptor(s) are very likely also involved in mediating HCV infection of PBMC subsets.

The principal site of HCV replication *in vivo* is within hepatocytes; infection of haematopoietic cells occurs at a relatively low level, and was at first controversial. Infection may be inhibited at many steps within the viral life-cycle, but experiments

carried out using retroviral particles pseudotyped with HCV E1E2, which have recently become available as a tool to investigate the HCV infection and entry process, suggest that this may be at least one of the stages at which HCV infection of PBMC subsets is restricted. None of the studies using pseudotyped particles found them to be capable of infecting PBMCs (Bartosch *et al.*, 2003a; Cormier *et al.*, 2004; Zhang *et al.*, 2004). As discussed in chapter 3, this could be due to technical limitations in the utility of retrovirus-based pseudoparticles for determining the infectivity of non-dividing cells from blood. In addition, there are differences between recombinant HCV particles and native virions. For example, recombinant particles cannot directly bind to LDL and HDL (Lambot *et al.*, 2002; Voisset *et al.*, 2005; Wunschmann *et al.*, 2000), whereas most native virions from HCV infected patients are associated with lipoproteins (Agnello *et al.*, 1999; Andre *et al.*, 2002). Bartosch *et al.* recently reported that addition of normal human serum or HDL during the production of pseudoparticles increased their infectivity for Huh-7 cells (Bartosch *et al.*, 2005). Such differences may mean that infectivity of pseudoparticles may not reflect the infectivity of HCV virions *in vivo*.

Alternatively, PBMCs may not express all the receptor(s) which are involved in mediating efficient HCV infection of hepatocytes, and/or may express suboptimal levels of key receptors. Notably, hepatic cells express high level of SR-BI, whilst my results suggest that the level of SR-BI expressed on monocytes and DCs may be relatively low. SR-BI expression levels may thus be at least one of the factors that limits the efficiency with which HCV pseudotyped particles and/or virions infect PBMCs.

It is also plausible that HCV utilises different mechanisms to enter haematopoietic cells and cells in the liver. For example, anti-HCV envelope Abs may facilitate virion binding to certain PBMC subsets through Fc receptors, and HCV may be internalised via these receptors with or without help from HCV-specific receptors such as CD81 and

SR-BI. This phenomenon of Ab-mediated enhancement of viral infectivity has been described in dengue virus infection (Littaua *et al.*, 1990; Peiris & Porterfield, 1979). HCV-specific B cells may also bind virions by means of surface immunoglobulins and then internalise viral particles attached in this way, a mechanism by which LCMV has been suggested to infect B cells producing anti-viral Abs (Planz *et al.*, 1996). Employment of this type of mechanism to infect immune cells would be consistent with the observation that APCs and B cells are the cell types reported to be infected by HCV *in vivo*. Had time permitted, it would have been of interest to address whether addition of anti-E2 or anti-E1 Abs enhances the infectivity of pseudoparticles expressing HCV E1E2 for APCs which express Fc receptors.

One of the motives for studying virus-receptor interactions is to provide information that will facilitate the design of antiviral agents that target the virion attachment/entry process and vaccines aiming to elicit neutralising Abs that block virus attachment/entry. For example, HIV-entry inhibitors targeting chemokine co-receptors have been developed and a fusion inhibitor (the gp41-derived synthetic peptide T20) is already in clinical use (Santoro *et al.*, 2004). Many vaccines work through eliciting neutralising Abs e.g. the recombinant hepatitis B surface antigen vaccine for HBV. HCV neutralising Abs should ideally be potently neutralising and target epitopes conserved across different HCV genotypes/subtypes; they should also be difficult for the virus to mutate to evade. Simultaneously, it is important that these neutralising Abs do not enhance viral infectivity.

Definition of receptor-binding sites on viral attachment proteins can give insight into epitopes that molecules can target to block the interaction, or Abs can bind to mediate viral neutralisation.

HVR-1 within E2 plays several roles in the HCV life-cycle: it is responsible for E2 binding to SR-BI (Bartosch *et al.*, 2003b; Scarselli *et al.*, 2002) and GAGs (Basu *et al.*, 2004; Yagnik *et al.*, 2000); it is targeted by most neutralising Abs (Farci *et al.*, 1996); and it enables HCV to escape immune responses by undergoing mutation and via its ability to mimic immunoglobulin structure (Hu *et al.*, 2005). HVR-1 always exists in infectious clones, suggesting that the presence of this region has a survival advantage. In support of this, it has been published that the presence of HVR-1 enhances the infectivity of pseudotyped particles expressing HCV E1E2 with HDL in a SR-BI dependent manner (Bartosch *et al.*, 2005). A HCV clone lacking HVR-1 was shown to be infectious in a chimpanzee model; but variants with point mutations in E2 were found to have appeared and rescued the deletion of HVR-1 (Forns *et al.*, 2000; Scarselli *et al.*, 2002). It is thought that HVR-1 is not randomly variable, but maintains a certain conformation. Indeed, mimotopes that targeted the conserved conformational structure of HVR-1 were able to raise Abs with extensive cross-reactivity (Roccasecca *et al.*, 2001). Importantly, neutralising Abs directed against HVR-1 have been found to be protective in the chimpanzee model (Esumi *et al.*, 1999; Farci *et al.*, 1994; Farci *et al.*, 1996). However, Lavillette *et al.* reported that HCV E1E2s derived from various genotypes/subtypes exhibit a differential capacity to interact with SR-BI, suggesting that alternative receptors may be used by different genotypes/subtypes (Lavillette *et al.*, 2005a). This suggests that it may be advantageous to target epitopes in the HCV envelope proteins outside of HVR-1 to raise broadly-neutralising Abs. Interestingly, the CD81 binding sites within E2 are thought to be outside of HVR-1, as discussed in chapter 3. CD81 is currently the only receptor known to be absolutely required for the infection of hepatocytes by HCVs from all genotypes. I found in this study that the pattern of binding of soluble E2 proteins from different strains to PBMC subsets was

correlated with their level of CD81 expression, suggesting the importance of E2-CD81 interaction in binding to PBMC subsets. Thus E2-CD81 interaction may be a good target for the development of antivirals/vaccine that may have activity against HCVs from all genotypes/subtypes. As discussed later, E2-CD81 interaction has also been shown to modulate immune functions. Thus blocking E2-CD81 interaction may prevent or reduce the severity of HCV-related pathogenesis promoted by immunomodulatory effects of E2. Blocking agents targeting this interaction could potentially be used in combination with existing anti-viral therapies.

Despite the discovery of several putative receptors that E2 can interact with, it is not clear whether there are additional host cell surface components that may act as HCV receptors/co-receptors. It is also not clear what combinations of known receptors/co-receptors are required for HCV infection of different cell types. These questions need to be answered if anti-viral agents are to be developed to target the HCV attachment/entry process. Determination of the crystal structure of E2 or an E2-CD81 complex would also be helpful to facilitate the design of neutralising Abs or molecules to block this interaction. This would give insight into the E2-CD81 interaction sites within the LEL of CD81 and the E2 protein, and may help to provide a better understanding of the multi-step process of HCV entry. Additionally, structural studies may also lead to the identification of natural ligands for CD81. However crystallisation studies have been hampered by difficulties including production and purification of sufficient quantities of soluble monomeric E2 protein, and the fact that E2 is a heavily glycosylated protein.

In chapter 3, I showed that soluble E2 proteins from different HCV strains bound to PBMCs with differing efficiency, with the level of PBMC binding paralleling the affinity of binding of the different soluble E2 proteins to CD81. E2s derived from the

H77 strain are to date the only E2s that have been shown to have a very high CD81 binding affinity. It is unclear what impact if any, differences in E2-CD81 binding affinity may have on the infectivity of viruses bearing these proteins. HCV pseudotyped particles expressing E1E2 proteins from viruses whose E2s have low and high CD81 binding affinity have been shown to be infectious in *in vitro* systems (Bartosch *et al.*, 2003b; Lavillette *et al.*, 2005a; McKeating *et al.*, 2004; Zhang *et al.*, 2004), although difficulties in standardising the level of HCV protein expression on different particle preparations make it hard to perform accurate comparisons of the relative levels of infectivity conferred by different E1E2s. Differences in the CD81 binding affinity of E2 proteins may also affect their immunomodulatory properties. The immunomodulatory effects of E2-CD81 interaction that have been reported were all described in studies using the H77c E2 protein, which has a high CD81 binding ability. E2s from other genotypes/strains with low CD81 binding affinity may thus not be able to mediate the same effects as H77c E2. The affinity of anti-CD81 mAb clone JS-81 for CD81 is stronger than that of H77c E2 (Rosa *et al.*, 1996; Soldaini *et al.*, 2003). However the effect of E2 in the context of E1E2 heterodimers on native virions may be different from that of a plate-bound array of E2. Dissociation of E1E2 heterodimers from CD81 has been shown to be slower than that of soluble E2, implying that E1E2 may have a prolonged ability to interact with cells, and may have a more potent effect (Cocquerel *et al.*, 2003; Nakajima *et al.*, 2005). This raises the possibility that heterodimers of E1E2 on virions from strains with only weak CD81 binding affinity may bind sufficiently well to CD81 to induce immunomodulatory effects on immune cells. In support of this, there have not been any studies that have documented a correlation between infection with HCVs with differential CD81 binding ability and the persistence of virus or the development of the various pathologies that HCV infection is associated with. Further

studies are thus required to understand the importance of the differential binding of HCV E2s to lymphocyte subsets as a determinant of the *in vivo* disease course.

The interaction of E2 with CD81 on host cells could potentially affect their functions either by blocking or by mimicking the natural activities of CD81. CD81 is broadly expressed and likely has multiple functions on different immune system cell types. Its functions on T and B cells have been most extensively characterised. It has been reported that mAb ligation of CD81 lowers the threshold for T and B cell activation induced by signal transduction through the TCR or BCR respectively (Fearon & Carter, 1995; Imai & Yoshie, 1993; Mannion *et al.*, 1996; Wack *et al.*, 2001). Accordingly, cross-linking of CD81 on T cells by plate-bound soluble E2s has been shown to induce a co-stimulatory signal that enhances TCR-mediated proliferation and cytokine production (Wack *et al.*, 2001). This may allow priming of T cells that receive sub-optimal stimuli, enhance the clonal expansion of T cells in lymph nodes and result in the infiltration of activated T cells into the liver, which in turn may promote T cell-mediated damage in the liver.

Cross-linking of CD81 on B cells by E2s has also been shown to deliver a co-stimulatory signal that could promote activation, proliferation and Ab production (Cocquerel *et al.*, 2003; Rosa, 2001). Machida *et al.* also found that E2-CD81 interaction enhanced expression of activation-induced cytidine deaminase (AID) and induced hypermuation of the V_H gene in B cells (Machida *et al.*, 2005). Since AID promotes DNA recombination and somatic hypermutation, the over-expression of AID may be oncogenic (Okazaki *et al.*, 2003), which may lead to HCV-associated B cell lymphoproliferative diseases. It is also possible that polyclonal activation of B cells may

contribute to the delayed seroconversion observed in HCV infection; as has been proposed in LCMV infection of mice (Recher *et al.*, 2004).

At the time this project was started, nothing was known about functions of CD81 on cells of the innate immune system. Since NK cells expressed high levels of CD81 and the HCV E2 protein could bind to NK cells, I investigated the role of CD81 on NK cell responses. During my PhD, it was reported that cross-linking of CD81 on NK cells by mAbs or by E2 resulted in the inhibition of NK cell cytotoxicity, proliferation and production of cytokines (IFN-γ and TNF-α) (Crotta *et al.*, 2002; Tseng & Klimpel, 2002). By contrast, I found that NK cell activation in response to stimulation including anti-CD16 mAb and cytokines was not specifically inhibited by mAb cross-linking of CD81. However, I did obtain some data to suggest mAb cross-linking of CD81 inhibited the activation of NK cells via NKG2D in the presence of IL-2 (chapter 4). These results suggest a possible role for CD81 in the modulation of signalling through NKG2D. CD81 may modulate the membrane organisation of molecules required for inhibitory/stimulatory receptor signalling on NK cells. Had time permitted, it would have been of interest to address whether E2 (soluble E2 or E2 in the form of E1E2 complexes) was able to block or mimic this activity.

NK cells play an important effector role in controlling many viral infections especially in the early phase, and also have immunoregulatory functions. The importance of NK cells in HCV infection has been supported by the finding that homozygosity for HLA-C1 and KIR2DL3 is associated with resolution of HCV infection (Khakoo *et al.*, 2004; Parham, 2004). Thus it is not surprising that HCV may have evolved mechanisms for evading control by NK cells.

Defects in NK cell numbers and functions have been reported in HCV infection, for example, patients chronically infected with HCV have been shown to have a reduced frequency of NK cells with decreased cytotoxic activity (Corado *et al.*, 1997; Gabrielli *et al.*, 1995). This is well correlated with the fact that patients with chronic HCV infection have altered proportions of $CD56^{dim}$ and $CD56^{high}$ subsets, which may result in the alteration of the functional properties of the total peripheral blood NK population (Meier *et al.*, 2005). Although the mechanisms responsible for the altered NK cell subset composition are not known, it could be partly explained by reduced production of cytokines involved in NK cell homeostasis e.g. IL-15 (Meier *et al.*, 2005). This may be a common phenomenon in chronic virus infections, since an altered NK subset composition and reduced serum IL-15 levels were also observed in chronic HIV infection (Meier *et al.*, 2005).

Many viruses down-regulate surface MHC class I expression on the cells they infect to evade class I-restricted T cell killing. In turn, this renders the cells more susceptible to NK killing. Thus some viruses have evolved strategies for avoiding NK cell recognition by selectively inducing expression of ligands for NK inhibitory receptors on the cells they infect. For example, human CMV expresses UL18, a viral MHC class I mimic, which inhibits NK cell activation by interaction with the inhibitory NK receptor LIR-1 (Cosman *et al.*, 1997). Murine CMV and rat CMV also encode MHC class I homologues to inhibit NK cells via ligation of inhibitory receptors (Farrell *et al.*, 1997; Kloover *et al.*, 2002; Kubota *et al.*, 1999). The strategy employed by HIV is slightly different from CMV; HIV selectively down-regulates HLA-A and HLA-B, which present many viral peptides, on the cells it infects, but maintains the expression of HLA-C and HLA-E to inhibit NK activity (Cohen *et al.*, 1999). Notably, intrahepatic

expression of HLA-E, which can inhibit NK cell cytotoxicity through NKG2A, was found to be up-regulated in chronic HCV patients (Nattermann *et al.*, 2005).

In addition, it has been reported that the frequency of NK cells and $CD8^+$ T cells expressing the inhibitory receptor NKG2A is higher in chronic HCV patients than normal controls (Jinushi *et al.*, 2004; Nattermann *et al.*, 2003; Nattermann *et al.*, 2005). A high frequency of $NKG2A^+$ $CD8^+$ T cells has also been observed in HIV-infected patients and mice infected with different viruses (De Maria & Moretta, 2000; Kambayashi *et al.*, 2000; McMahon *et al.*, 2002; Moser *et al.*, 2002), while the frequency of NK and T cells expressing the activating receptor NKG2C is higher in CMV-infected patients than normal controls (Guma *et al.*, 2004). It has also been shown that $NKG2C^+$ NK cells do not co-express NKG2A (Guma *et al.*, 2004). In addition, alterations in the balance of inhibitory and activating receptor expression on NK cells have been observed in viremic HIV-positive patients, with expression of the natural cytotoxicity receptors NKp46, NKp30 and NKp44 on NK cells being reduced (De Maria *et al.*, 2003). These findings indicate that there may be differential selection of NK and T cells expressing activating versus inhibitory receptors during the course of different virus infections. Thus it is plausible that during HCV infection, the balance of regulatory receptor expression on NK cells may be shifted towards expression of inhibitory receptors.

If E2-CD81 interaction does inhibit NK cell activation via activating receptors, this may synergise with these mechanisms to produce a profound reduction in NK effector activities, potentially contributing to the induction and maintenance of HCV persistence. However further work is required to clarify the importance of E2-CD81 interaction in modulating NK cell responses *in vivo*.

DCs also play an important role in orchestrating innate and adaptive immunity. Many viruses have evolved strategies for inducing loss or functional impairment of these cells to promote persistence in their hosts. There have not been any reports of DC activation and functions during acute HCV infection. A reduced frequency of myeloid and/or plasmacytoid DCs has been found in the periphery of chronic HCV patients by some (Anthony *et al.*, 2004; Kanto *et al.*, 2004) but not all groups (Piccioli *et al.*, 2005). Chronic HCV infection has also been shown to adversely affect the production of IL-12, IFN-γ and IFN-α by DCs (Anthony *et al.*, 2004; Kanto *et al.*, 1999; Kanto *et al.*, 2004) and the allostimulatory function of DCs (Auffermann-Gretzinger *et al.*, 2001; Bain *et al.*, 2001; Kanto *et al.*, 1999; Kanto *et al.*, 2004). Moreover, DCs from HCV infected patients have been reported to exhibit impairments in maturation in response to stimuli, maintaining an immature phenotype as characterised by surface marker expression and continued capacity to uptake antigen (Auffermann-Gretzinger *et al.*, 2001; Kanto *et al.*, 1999). Again, however, not all groups have observed this (Longman *et al.*, 2004; Piccioli *et al.*, 2005; Rollier *et al.*, 2003). Inadequate T cell responses are also found in HCV patients with chronic infection, which may result from impaired functions of DCs. Although the exact mechanisms involved in impairment of DC function are not clear, it has been reported that the HCV core protein can modulate functions of DCs (Auffermann-Gretzinger *et al.*, 2001; Dolganiuc *et al.*, 2003; Hiasa *et al.*, 1998; Kim *et al.*, 2002; Lee *et al.*, 2001; Sarobe *et al.*, 2002); this has been associated with the observed defects in DCs from chronic HCV patients in some but not all studies.

In chapter 3, I showed that myeloid DCs, plasmacytoid DCs and monocyte-derived DCs expressed CD81 and that the HCV E2 protein could bind to these cells via both CD81-dependent and CD81-independent mechanisms. It is thus possible that interaction of E2 with receptor proteins on DCs may modulate their activation and functions. The

functions of CD81 on DCs are not known, thus I investigated the effect of cross-linking of CD81 on DCs on their response to different stimuli. MAb cross-linking of CD81 was not found to have any effect on the phenotypic and functional activation of monocyte-derived DCs in response to stimulation with LPS, poly(I:C) or an anti-CD40 mAb (chapter 5). In addition, bone-marrow-derived DCs from CD81-deficient mice responded normally to stimulation with LPS, poly(I:C) and an anti-CD40 mAb. However, it is possible that CD81 cross-linking may modulate functions of DCs other than those investigated in this study, e.g. migration or antigen presentation, as discussed in chapter 5.

It is also possible that HCV may alter the functions of DCs through E2 interaction with other receptors such as DC-SIGN or SR-BI. Several microorganisms use DC-SIGN to enhance their dissemination and modulate DC responses (Andrews *et al.*, 2001; Raftery *et al.*, 2004; Sol-Foulon *et al.*, 2002). One possible role of HCV binding to DC-SIGN on DCs may be in delivery of HCV to the liver. A common feature of the pathogens that interact with DC-SIGN is that they cause chronic infections that may last a life-time and also that manipulation of the Th1/Th2 balance is central to their persistence (Appelmelk *et al.*, 2003). It has been reported that the interaction between mycobacterial mannosylated lipoarabinomannan and DC-SIGN, which is mediated by the same binding site as that involved in HCV glycoprotein binding to DC-SIGN, can modulate the response of DCs to LPS (Geijtenbeek *et al.*, 2003). The interaction was found to reduce the LPS-induced maturation of DCs and IL-12 production. In contrast, it enhanced the production of the anti-inflammatory cytokine IL-10, shifting the immune response in a Th2 direction, which results in immune evasion and promotion of pathogen persistence. It is thus possible that interaction between E2 and DC-SIGN may modulate the response of DCs during HCV infection as in mycobacterial infection.

SR-BI has been reported to be involved in innate immune responses by acting as a pattern recognition receptor, particularly on monocytes and macrophages (Bocharov *et al.*, 2004a; Imachi *et al.*, 2000; Murao *et al.*, 1997; Pearson, 1996; Vishnyakova *et al.*, 2003). When incubated with human plasma, the majority of LPS associates with lipoproteins, especially with HDL (de Haas *et al.*, 2000; Levels *et al.*, 2001). Interestingly, SR-BI can bind and internalise both lipoprotein-free, monomerised LPS and HDL-associated LPS (Vishnyakova *et al.*, 2003), and thus plays an important role in the clearance of LPS from plasma. It has been shown that LPS competes with HDL and apolipoproteins for binding to SR-BI, indicating that lipid transport and LPS neutralisation utilise similar mechanisms to enter the cells via SR-BI (Bocharov *et al.*, 2004a). Since most HCV virions from HCV infected patients are associated with lipoproteins (Agnello *et al.*, 1999; Andre *et al.*, 2002), HCV may utilise SR-BI for its entry in a manner similar to that via which LPS or cholesterol are internalised. In this case, HCV interaction with SR-BI may block or enhance LPS uptake or cholesterol uptake/efflux via SR-BI. In addition, SR-BI may regulate the surface clustering of LPS/membrane CD14 and TLR-4 (Babitt *et al.*, 1997; Graf *et al.*, 1999; Vishnyakova *et al.*, 2003). E2 binding may thus potentially affect LPS signalling via SR-BI. Had time permitted, it would have been of interest to address whether HCV E2 can modulate DC responses through SR-BI.

In summary, the interaction of the HCV E2 protein with host cell surface receptors may have two roles in virus infection: enabling the virus to enter the host cells, and modulating immune responses. More studies are required to understand the mechanisms involved in HCV infection of PBMC subsets, and the role this may play in determining the course of HCV infection. Additionally, much more work is required to elucidate

whether/how interaction of the HCV glycoproteins with receptors (including CD81) on immune system cell subsets may mediate immunomodulatory effects *in vivo*. Clarification of these issues may help to facilitate the design of therapeutic strategies to combat HCV infection and reduce the immunopathological consequences of E2-receptor interaction.

References

Abbate, I., Cappiello, G., Lo Iacono, O., Longo, R., Ferraro, D., Antonucci, G., Di Marco, V., Di Stefano, R., Craxi, A., Solmone, M. C., Spano, A., Ippolito, G. & Capobianchi, M. R. (2003). Heterogeneity of HVR-1 quasispecies is predictive of early but not sustained virological response in genotype 1b-infected patients undergoing combined treatment with PEG- or STD-IFN plus RBV. *J Biol Regul Homeost Agents* **17**, 162-165.

Acton, S., Rigotti, A., Landschulz, K. T., Xu, S., Hobbs, H. H. & Krieger, M. (1996). Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science* **271**, 518-520.

Adams, N. J., Chamberlain, R. W., Taylor, L. A., Davidson, F., Lin, C. K., Elliott, R. M. & Simmonds, P. (1997). Complete coding sequence of hepatitis C virus genotype 6a. *Biochem Biophys Res Commun* **234**, 393-396.

Agnello, V., Chung, R. T. & Kaplan, L. M. (1992). A role for hepatitis C virus infection in type II cryoglobulinemia. *N Engl J Med* **327**, 1490-1495.

Agnello, V., Abel, G., Elfahal, M., Knight, G. B. & Zhang, Q. X. (1999). Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proc Natl Acad Sci USA* **96**, 12766-12771.

Alcami, A. (2003). Viral mimicry of cytokines, chemokines and their receptors. *Nat Rev Immunol* **3**, 36-50.

Alexopoulou, L., Holt, A. C., Medzhitov, R. & Flavell, R. A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413**, 732-738.

Alimonti, J. B., Ball, T. B. & Fowke, K. R. (2003). Mechanisms of CD4+ T lymphocyte cell death in human immunodeficiency virus infection and AIDS. *J Gen Virol* **84**, 1649-1661.

Allander, T., Forns, X., Emerson, S. U., Purcell, R. H. & Bukh, J. (2000). Hepatitis C virus envelope protein E2 binds to CD81 of tamarins. *Virology* **277**, 358-367.

Alter, H. J. (1995). To C or not to C: these are the questions. *Blood* **85**, 1681-1695.

Alter, H. J., Sanchez-Pescador, R., Urdea, M. S., Wilber, J. C., Lagier, R. J., Di Bisceglie, A. M., Shih, J. W. & Neuwald, P. D. (1995). Evaluation of branched DNA signal amplification for the detection of hepatitis C virus RNA. *J Viral Hepat* **2**, 121-132.

Alter, M. J. (1997). Epidemiology of hepatitis C. *Hepatology* **26**, 62S-65S.

Andersson, M., Paabo, S., Nilsson, T. & Peterson, P. A. (1985). Impaired intracellular transport of class I MHC antigens as a possible means for adenoviruses to evade immune surveillance. *Cell* **43**, 215-222.

Ando, K., Hiroishi, K., Kaneko, T., Moriyama, T., Muto, Y., Kayagaki, N., Yagita, H., Okumura, K. & Imawari, M. (1997). Perforin, Fas/Fas ligand, and TNF-alpha pathways as specific and bystander killing mechanisms of hepatitis C virus-specific human CTL. *J Immunol* **158**, 5283-5291.

Andre, P., Komurian-Pradel, F., Deforges, S., Perret, M., Berland, J. L., Sodoyer, M., Pol, S., Brechot, C., Paranhos-Baccala, G. & Lotteau, V. (2002). Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. *J Virol* **76**, 6919-6928.

Andre, P., Castriconi, R., Espeli, M., Anfossi, N., Juarez, T., Hue, S., Conway, H., Romagne, F., Dondero, A., Nanni, M., Caillat-Zucman, S., Raulet, D. H., Bottino, C., Vivier, E., Moretta, A. & Paul, P. (2004). Comparative analysis of human NK cell activation induced by NKG2D and natural cytotoxicity receptors. *Eur J Immunol* **34**, 961-971.

Andrews, D. M., Andoniou, C. E., Granucci, F., Ricciardi-Castagnoli, P. & Degli-Esposti, M. A. (2001). Infection of dendritic cells by murine cytomegalovirus induces functional paralysis. *Nat Immunol* **2**, 1077-1084.

Anthony, D. D., Yonkers, N. L., Post, A. B., Asaad, R., Heinzel, F. P., Lederman, M. M., Lehmann, P. V. & Valdez, H. (2004). Selective impairments in dendritic cell-associated function distinguish hepatitis C virus and HIV infection. *J Immunol* **172**, 4907-4916.

Appay, V., Dunbar, P. R., Callan, M., Klenerman, P., Gillespie, G. M., Papagno, L., Ogg, G. S., King, A., Lechner, F., Spina, C. A., Little, S., Havlir, D. V., Richman, D. D., Gruener, N., Pape, G., Waters, A., Easterbrook, P., Salio, M., Cerundolo, V., McMichael, A. J. & Rowland-Jones, S. L. (2002). Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med* **8**, 379-385.

Appelmelk, B. J., van Die, I., van Vliet, S. J., Vandenbroucke-Grauls, C. M., Geijtenbeek, T. B. & van Kooyk, Y. (2003). Cutting edge: carbohydrate profiling identifies new pathogens that interact with dendritic cell-specific ICAM-3-grabbing nonintegrin on dendritic cells. *J Immunol* **170**, 1635-1639.

Arenas, J. I., Gallegos-Orozco, J. F., Laskus, T., Wilkinson, J., Khatib, A., Fasola, C., Adair, D., Radkowski, M., Kibler, K. V., Nowicki, M., Douglas, D., Williams, J., Netto, G., Mulligan, D., Klintmalm, G., Rakela, J. & Vargas, H. E. (2004). Hepatitis C virus quasi-species dynamics predict progression of fibrosis after liver transplantation. *J Infect Dis* **189**, 2037-2046. Epub 2004 May 2010.

Arnon, T. I., Lev, M., Katz, G., Chernobrov, Y., Porgador, A. & Mandelboim, O. (2001). Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur J Immunol* **31**, 2680-2689.

Ashkar, A. A. & Rosenthal, K. L. (2003). Interleukin-15 and natural killer and NKT cells play a critical role in innate protection against genital herpes simplex virus type 2 infection. *J Virol* **77**, 10168-10171.

Asselin-Paturel, C., Boonstra, A., Dalod, M., Durand, I., Yessaad, N., Dezutter-Dambuyant, C., Vicari, A., O'Garra, A., Biron, C., Briere, F. & Trinchieri, G. (2001). Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat Immunol* **2**, 1144-1150.

Auffermann-Gretzinger, S., Keeffe, E. B. & Levy, S. (2001). Impaired dendritic cell maturation in patients with chronic, but not resolved, hepatitis C virus infection. *Blood* **97**, 3171-3176.

Azzoni, L., Zatsepina, O., Abebe, B., Bennett, I. M., Kanakaraj, P. & Perussia, B. (1998). Differential transcriptional regulation of CD161 and a novel gene, 197/15a, by IL-2, IL-15, and IL-12 in NK and T cells. *J Immunol* **161**, 3493-3500.

Babitt, J., Trigatti, B., Rigotti, A., Smart, E. J., Anderson, R. G., Xu, S. & Krieger, M. (1997). Murine SR-BI, a high density lipoprotein receptor that mediates selective lipid uptake, is N-glycosylated and fatty acylated and colocalizes with plasma membrane caveolae. *J Biol Chem* **272**, 13242-13249.

Bain, C., Fatmi, A., Zoulim, F., Zarski, J. P., Trepo, C. & Inchauspe, G. (2001). Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. *Gastroenterology* **120**, 512-524.

Ballare, M., Airoldi, G., Brunetto, M. R., Manzini, P., Bordin, G., Touscoz, A., Bonino, F. & Monteverde, A. (1993). Hepatitis C virus infection in type II essential mixed cryoglobulinemias. *Arch Virol Suppl* **8**, 113-121.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B. & Palucka, K. (2000). Immunobiology of dendritic cells. *Annu Rev Immunol* **18**, 767-811.

Bancroft, G. J., Shellam, G. R. & Chalmer, J. E. (1981). Genetic influences on the augmentation of natural killer (NK) cells during murine cytomegalovirus infection: correlation with patterns of resistance. *J Immunol* **126**, 988-994.

Bancu, A. C., Gherman, M., Sulica, A., Goto, T., Farrar, W. & Herberman, R. B. (1988). Regulation of human natural cytotoxicity by IgG. II. Cyclic AMP as a mediator of monomeric IgG-induced inhibition of natural killer cell activity. *Cell Immunol* **114**, 246-256.

Banki, Z., Kacani, L., Mullauer, B., Wilflingseder, D., Obermoser, G., Niederegger, H., Schennach, H., Sprinzl, G. M., Sepp, N., Erdei, A., Dierich, M. P. & Stoiber, H. (2003). Cross-linking of CD32 induces maturation of human monocyte-derived dendritic cells via NF-kappa B signaling pathway. *J Immunol* **170**, 3963-3970.

Baribaud, F., Pohlmann, S., Leslie, G., Mortari, F. & Doms, R. W. (2002). Quantitative expression and virus transmission analysis of DC-SIGN on monocyte-derived dendritic cells. *J Virol* **76**, 9135-9142.

Bartenschlager, R. & Lohmann, V. (2000). Replication of the hepatitis C virus. *Baillieres Best Pract Res Clin Gastroenterol* **14**, 241-254.

Barth, H., Cerino, R., Arcuri, M., Hoffmann, M., Schurmann, P., Adah, M. I., Gissler, B., Zhao, X., Ghisetti, V., Lavezzo, B., Blum, H. E., von Weizsacker, F., Vitelli, A., Scarselli, E. & Baumert, T. F. (2005). Scavenger receptor class B type I and hepatitis C virus infection of primary tupaia hepatocytes. *J Virol* **79**, 5774-5785.

Bartosch, B., Dubuisson, J. & Cosset, F. L. (2003a). Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *Journal of Experimental Medicine* **197**, 633-642.

Bartosch, B., Verney, G., Dreux, M., Donot, P., Morice, Y., Penin, F., Pawlotsky, J. M., Lavillette, D. & Cosset, F. L. (2005). An interplay between hypervariable region 1 of the hepatitis C virus E2 glycoprotein, the scavenger receptor BI, and high-density lipoprotein promotes both enhancement of infection and protection against neutralizing antibodies. *J Virol* **79**, 8217-8229.

Bartosch, B., Vitelli, A., Granier, C., Goujon, C., Dubuisson, J., Pascale, S., Scarselli, E., Cortese, R., Nicosia, A. & Cosset, F. L. (2003b). Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. *Journal of Biological Chemistry* **278**, 41624-41630.

Bashirova, A. A., Geijtenbeek, T. B., van Duijnhoven, G. C., van Vliet, S. J., Eilering, J. B., Martin, M. P., Wu, L., Martin, T. D., Viebig, N., Knolle, P. A., KewalRamani, V. N., van Kooyk, Y. & Carrington, M. (2001). A dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN)-related protein is highly expressed on human liver sinusoidal endothelial cells and promotes HIV-1 infection. *J Exp Med* **193**, 671-678.

Bassett, S. E., Thomas, D. L., Brasky, K. M. & Lanford, R. E. (1999). Viral persistence, antibody to E1 and E2, and hypervariable region 1 sequence stability in hepatitis C virus-inoculated chimpanzees. *J Virol* **73**, 1118-1126.

Basu, A., Beyene, A., Meyer, K. & Ray, R. (2004). The hypervariable region 1 of the e2 glycoprotein of hepatitis C virus binds to glycosaminoglycans, but this binding does not lead to infection in a pseudotype system. *J Virol* **78**, 4478-4486.

Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J. H., Lanier, L. L. & Spies, T. (1999). Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* **285**, 727-729.

Baumert, T. F., Ito, S., Wong, D. T. & Liang, T. J. (1998). Hepatitis C virus structural proteins assemble into viruslike particles in insect cells. *J Virol* **72**, 3827-3836.

Beard, M. R., Abell, G., Honda, M., Carroll, A., Gartland, M., Clarke, B., Suzuki, K., Lanford, R., Sangar, D. V. & Lemon, S. M. (1999). An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. *Hepatology* **30**, 316-324.

Belkaid, Y. & Rouse, B. T. (2005). Natural regulatory T cells in infectious disease. *Nat Immunol* **6**, 353-360.

Berard, M., Brandt, K., Bulfone-Paus, S. & Tough, D. F. (2003). IL-15 promotes the survival of naive and memory phenotype CD8+ T cells. *J Immunol* **170**, 5018-5026.

Berditchevski, F., Tolias, K. F., Wong, K., Carpenter, C. L. & Hemler, M. E. (1997). A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. *J Biol Chem* **272**, 2595-2598.

Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J. & Zako, M. (1999). Functions of cell surface heparan sulfate proteoglycans. *Annu Rev Biochem* **68**, 729-777.

Bigger, C. B., Brasky, K. M. & Lanford, R. E. (2001). DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection. *J Virol* **75**, 7059-7066.

Billadeau, D. D., Upshaw, J. L., Schoon, R. A., Dick, C. J. & Leibson, P. J. (2003). NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. *Nat Immunol* **4**, 557-564.

Biron, C. A. (2001). Interferons alpha and beta as immune regulators--a new look. *Immunity* **14**, 661-664.

Biron, C. A. & Brossay, L. (2001). NK cells and NKT cells in innate defense against viral infections. *Curr Opin Immunol* **13**, 458-464.

Biron, C. A., Byron, K. S. & Sullivan, J. L. (1989). Severe herpesvirus infections in an adolescent without natural killer cells. *N Engl J Med* **320**, 1731-1735.

Biron, C. A., Nguyen, K. B. & Pien, G. C. (2002). Innate immune responses to LCMV infections: natural killer cells and cytokines. *Curr Top Microbiol Immunol* **263**, 7-27.

Biron, C. A., Nguyen, K. B., Pien, G. C., Cousens, L. P. & Salazar-Mather, T. P. (1999). Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol* **17**, 189-220.

Blight, K., Lesniewski, R. R., LaBrooy, J. T. & Gowans, E. J. (1994). Detection and distribution of hepatitis C-specific antigens in naturally infected liver. *Hepatology* **20**, 553-557.

Blight, K. J., Kolykhalov, A. A. & Rice, C. M. (2000). Efficient initiation of HCV RNA replication in cell culture. *Science* **290**, 1972-1974.

Bocharov, A. V., Baranova, I. N., Vishnyakova, T. G., Remaley, A. T., Csako, G., Thomas, F., Patterson, A. P. & Eggerman, T. L. (2004a). Targeting of scavenger receptor class B type I by synthetic amphipathic alpha-helical-containing peptides blocks lipopolysaccharide (LPS) uptake and LPS-induced pro-inflammatory cytokine

responses in THP-1 monocyte cells. *J Biol Chem* **279**, 36072-36082. Epub 32004 Jun 36015.

Bocharov, G., Ludewig, B., Bertoletti, A., Klenerman, P., Junt, T., Krebs, P., Luzyanina, T., Fraser, C. & Anderson, R. M. (2004b). Underwhelming the immune response: effect of slow virus growth on CD8+-T-lymphocyte responses. *J Virol* **78**, 2247-2254.

Botero, R. C. (2004). Should patients with chronic hepatitis C infection be transplanted? *Transplant Proc* **36**, 1449-1454.

Boucheix, C. & Rubinstein, E. (2001). Tetraspanins. *Cell Mol Life Sci* **58**, 1189-1205.

Bouffard, P., Hayashi, P. H., Acevedo, R., Levy, N. & Zeldis, J. B. (1992). Hepatitis C virus is detected in a monocyte/macrophage subpopulation of peripheral blood mononuclear cells of infected patients. *J Infect Dis* **166**, 1276-1280.

Bourgeois, C., Rocha, B. & Tanchot, C. (2002). A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. *Science* **297**, 2060-2063.

Bradley, D. W., Maynard, J. E., Popper, H., Ebert, J. W., Cook, E. H., Fields, H. A. & Kemler, B. J. (1981). Persistent non-A, non-B hepatitis in experimentally infected chimpanzees. *J Infect Dis* **143**, 210-218.

Braud, V. M., Allan, D. S., O'Callaghan, C. A., Soderstrom, K., D'Andrea, A., Ogg, G. S., Lazetic, S., Young, N. T., Bell, J. I., Phillips, J. H., Lanier, L. L. & McMichael, A. J. (1998). HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* **391**, 795-799.

Breiman, A., Grandvaux, N., Lin, R., Ottone, C., Akira, S., Yoneyama, M., Fujita, T., Hiscott, J. & Meurs, E. F. (2005). Inhibition of RIG-I-dependent signaling to the interferon pathway during hepatitis C virus expression and restoration of signaling by IKKepsilon. *J Virol* **79**, 3969-3978.

Brown, M. G., Dokun, A. O., Heusel, J. W., Smith, H. R., Beckman, D. L., Blattenberger, E. A., Dubbelde, C. E., Stone, L. R., Scalzo, A. A. & Yokoyama, W. M. (2001). Vital involvement of a natural killer cell activation receptor in resistance to viral infection. *Science* **292**, 934-937.

Buechler, C., Ritter, M., Quoc, C. D., Agildere, A. & Schmitz, G. (1999). Lipopolysaccharide inhibits the expression of the scavenger receptor Cla-1 in human monocytes and macrophages. *Biochem Biophys Res Commun* **262**, 251-254.

Bukh, J., Miller, R. H. & Purcell, R. H. (1995). Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. *Semin Liver Dis* **15**, 41-63.

Burgert, H. G. & Kvist, S. (1985). An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class I antigens. *Cell* **41**, 987-997.

Cabrera, R., Tu, Z., Xu, Y., Firpi, R. J., Rosen, H. R., Liu, C. & Nelson, D. R. (2004). An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* **40**, 1062-1071.

Cacoub, P., Renou, C., Rosenthal, E., Cohen, P., Loury, I., Loustaud-Ratti, V., Yamamoto, A. M., Camproux, A. C., Hausfater, P., Musset, L., Veyssier, P., Raguin, G. & Piette, J. C. (2000). Extrahepatic manifestations associated with hepatitis C virus infection. A prospective multicenter study of 321 patients. The GERMIVIC. Groupe d'Etude et de Recherche en Medecine Interne et Maladies Infectieuses sur le Virus de l'Hepatite C. *Medicine (Baltimore)* **79**, 47-56.

Capobianchi, M. R., Abbate, I., Cappiello, G. & Solmone, M. (2003). HCV and interferon: viral strategies for evading innate defence mechanisms in the virus-host battle. *Cell Death Differ* **10**, S22-24.

Carlos, M. P., Yamamura, Y., Vu, Q., Conzen, K., Anderson, D. E. & Torres, J. V. (2004). Humoral immunity to immunodominant epitopes of Hepatitis C virus in individuals infected with genotypes 1a or 1b. *Clin Immunol* **111**, 22-27.

Carretero, M., Llano, M., Navarro, F., Bellon, T. & Lopez-Botet, M. (2000). Mitogen-activated protein kinase activity is involved in effector functions triggered by the CD94/NKG2-C NK receptor specific for HLA-E. *Eur J Immunol* **30**, 2842-2848.

Carson, W. E., Ross, M. E., Baiocchi, R. A., Marien, M. J., Boiani, N., Grabstein, K. & Caligiuri, M. A. (1995). Endogenous production of interleukin 15 by activated human monocytes is critical for optimal production of interferon-gamma by natural killer cells in vitro. *J Clin Invest* **96**, 2578-2582.

Carson, W. E., Fehniger, T. A., Haldar, S., Eckhert, K., Lindemann, M. J., Lai, C. F., Croce, C. M., Baumann, H. & Caligiuri, M. A. (1997). A potential role for interleukin-15 in the regulation of human natural killer cell survival. *J Clin Invest* **99**, 937-943.

Carson, W. E., Giri, J. G., Lindemann, M. J., Linett, M. L., Ahdieh, M., Paxton, R., Anderson, D., Eisenmann, J., Grabstein, K. & Caligiuri, M. A. (1994). Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J Exp Med* **180**, 1395-1403.

Carter, R. H. & Fearon, D. T. (1992). CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes. *Science* **256**, 105-107.

Cella, M., Facchetti, F., Lanzavecchia, A. & Colonna, M. (2000). Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. *Nat Immunol* **1**, 305-310.

Cella, M., Jarrossay, D., Facchetti, F., Alebardi, O., Nakajima, H., Lanzavecchia, A. & Colonna, M. (1999). Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat Med* **5**, 919-923.

Cerny, A. & Chisari, F. V. (1994). Immunological aspects of HCV infection. *Intervirology* **37**, 119-125.

Cerwenka, A. & Lanier, L. L. (2001). Ligands for natural killer cell receptors: redundancy or specificity. *Immunol Rev* **181**, 158-169.

Chamberlain, R. W., Adams, N. J., Taylor, L. A., Simmonds, P. & Elliott, R. M. (1997a). The complete coding sequence of hepatitis C virus genotype 5a, the predominant genotype in South Africa. *Biochem Biophys Res Commun* **236**, 44-49.

Chamberlain, R. W., Adams, N., Saeed, A. A., Simmonds, P. & Elliott, R. M. (1997b). Complete nucleotide sequence of a type 4 hepatitis C virus variant, the predominant genotype in the Middle East. *J Gen Virol* **78**, 1341-1347.

Chang, K. M., Rehermann, B., McHutchison, J. G., Pasquinelli, C., Southwood, S., Sette, A. & Chisari, F. V. (1997). Immunological significance of cytotoxic T lymphocyte epitope variants in patients chronically infected by the hepatitis C virus. *J Clin Invest* **100**, 2376-2385.

Charrin, S., Le Naour, F., Labas, V., Billard, M., Le Caer, J. P., Emile, J. F., Petit, M. A., Boucheix, C. & Rubinstein, E. (2003). EWI-2 is a new component of the tetraspanin web in hepatocytes and lymphoid cells. *Biochem J* **373**, 409-421.

Chayama, K., Tsubota, A., Kobayashi, M., Okamoto, K., Hashimoto, M., Miyano, Y., Koike, H., Koida, I., Arase, Y., Saitoh, S., Suzuki, Y., Murashima, N., Ikeda, K. & Kumada, H. (1997). Pretreatment virus load and multiple amino acid substitutions in the interferon sensitivity-determining region predict the outcome of interferon treatment in patients with chronic genotype 1b hepatitis C virus infection. *Hepatology* **25**, 745-749.

Chen, C. M., You, L. R., Hwang, L. H. & Lee, Y. H. (1997a). Direct interaction of hepatitis C virus core protein with the cellular lymphotoxin-beta receptor modulates the signal pathway of the lymphotoxin-beta receptor. *J Virol* **71**, 9417-9426.

Chen, M., Sallberg, M., Sonnerborg, A., Weiland, O., Mattsson, L., Jin, L., Birkett, A., Peterson, D. & Milich, D. R. (1999). Limited humoral immunity in hepatitis C virus infection. *Gastroenterology* **116**, 135-143.

Chen, Y., Maguire, T., Hileman, R. E., Fromm, J. R., Esko, J. D., Linhardt, R. J. & Marks, R. M. (1997b). Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat Med* **3**, 866-871.

Cherukuri, A., Shoham, T., Sohn, H. W., Levy, S., Brooks, S., Carter, R. & Pierce, S. K. (2004). The tetraspanin CD81 is necessary for partitioning of coligated CD19/CD21-B cell antigen receptor complexes into signaling-active lipid rafts. *J Immunol* **172**, 370-380.

Chinen, J. & Shearer, W. T. (2002). Molecular virology and immunology of HIV infection. *J Allergy Clin Immunol* **110**, 189-198.

Chinetti, G., Gbaguidi, F. G., Griglio, S., Mallat, Z., Antonucci, M., Poulain, P., Chapman, J., Fruchart, J. C., Tedgui, A., Najib-Fruchart, J. & Staels, B. (2000). CLA-1/SR-BI is expressed in atherosclerotic lesion macrophages and regulated by activators of peroxisome proliferator-activated receptors. *Circulation* **101**, 2411-2417.

Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. & Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**, 359-362.

Choo, Q. L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, R. & Barr, P. J. (1991). Genetic organization and diversity of the hepatitis C virus. *Proc Natl Acad Sci USA* **88**, 2451-2455.

Choukhi, A., Ung, S., Wychowski, C. & Dubuisson, J. (1998). Involvement of endoplasmic reticulum chaperones in the folding of hepatitis C virus glycoproteins. *J Virol* **72**, 3851-3858.

Chung, R. T., Monto, A., Dienstag, J. L. & Kaplan, L. M. (1999). Mutations in the NS5A region do not predict interferon-responsiveness in american patients infected with genotype 1b hepatitis C virus. *J Med Virol* **58**, 353-358.

Chung, Y. L., Sheu, M. L. & Yen, S. H. (2003). Hepatitis C virus NS5A as a potential viral Bcl-2 homologue interacts with Bax and inhibits apoptosis in hepatocellular carcinoma. *Int J Cancer* **107**, 65-73.

Cianciolo, G. J., Copeland, T. D., Oroszlan, S. & Snyderman, R. (1985). Inhibition of lymphocyte proliferation by a synthetic peptide homologous to retroviral envelope proteins. *Science* **230**, 453-455.

Clayton, R. F., Owsianka, A., Aitken, J., Graham, S., Bhella, D. & Patel, A. H. (2002). Analysis of antigenicity and topology of E2 glycoprotein present on recombinant hepatitis C virus-like particles. *J Virol* **76**, 7672-7682.

Cocquerel, L., Kuo, C. C., Dubuisson, J. & Levy, S. (2003). CD81-dependent binding of hepatitis C virus E1E2 heterodimers. *J Virol* **77**, 10677-10683.

Cocquerel, L., Meunier, J. C., Pillez, A., Wychowski, C. & Dubuisson, J. (1998). A retention signal necessary and sufficient for endoplasmic reticulum localization maps to the transmembrane domain of hepatitis C virus glycoprotein E2. *J Virol* **72**, 2183-2191.

Cocquerel, L., Wychowski, C., Minner, F., Penin, F. & Dubuisson, J. (2000). Charged residues in the transmembrane domains of hepatitis C virus glycoproteins play a major role in the processing, subcellular localization, and assembly of these envelope proteins. *J Virol* **74**, 3623-3633.

Cocquerel, L., Op de Beeck, A., Lambot, M., Roussel, J., Delgrange, D., Pillez, A., Wychowski, C., Penin, F. & Dubuisson, J. (2002). Topological changes in the transmembrane domains of hepatitis C virus envelope glycoproteins. *Embo J* **21**, 2893-2902.

Cognasse, F., Chavarin, P., Acquart, S., Sabido, O., Beniguel, L., Genin, C., Richard, Y. & Garraud, O. (2005). Differential downstream effects of CD40 ligation mediated by membrane or soluble CD40L and agonistic Ab: a study on purified human B cells. *Int J Immunopathol Pharmacol* **18**, 65-74.

Cohen, G. B., Gandhi, R. T., Davis, D. M., Mandelboim, O., Chen, B. K., Strominger, J. L. & Baltimore, D. (1999). The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity* **10**, 661-671.

Cohen, J. (1999). The scientific challenge of hepatitis C. *Science* **285**, 26-30.

Cole, G., Coleman, N. & Soilleux, E. (2004). HCV and HIV binding lectin, DC-SIGNR, is expressed at all stages of HCV induced liver disease. *J Clin Pathol* **57**, 79-80.

Conry-Cantilena, C., VanRaden, M., Gibble, J., Melpolder, J., Shakil, A. O., Viladomiu, L., Cheung, L., DiBisceglie, A., Hoofnagle, J., Shih, J. W. & et al. (1996). Routes of infection, viremia, and liver disease in blood donors found to have hepatitis C virus infection. *N Engl J Med* **334**, 1691-1696.

Cooper, M. A., Fehniger, T. A. & Caligiuri, M. A. (2001a). The biology of human natural killer-cell subsets. *Trends Immunol* **22**, 633-640.

Cooper, M. A., Fehniger, T. A., Turner, S. C., Chen, K. S., Ghaheri, B. A., Ghayur, T., Carson, W. E. & Caligiuri, M. A. (2001b). Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood* **97**, 3146-3151.

Cooper, M. A., Bush, J. E., Fehniger, T. A., VanDeusen, J. B., Waite, R. E., Liu, Y., Aguila, H. L. & Caligiuri, M. A. (2002). In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. *Blood* **100**, 3633-3638. Epub 2002 Jul 3635.

Cooper, S., Erickson, A. L., Adams, E. J., Kansopon, J., Weiner, A. J., Chien, D. Y., Houghton, M., Parham, P. & Walker, C. M. (1999). Analysis of a successful immune response against hepatitis C virus. *Immunity* **10**, 439-449.

Corado, J., Toro, F., Rivera, H., Bianco, N. E., Deibis, L. & De Sanctis, J. B. (1997). Impairment of natural killer (NK) cytotoxic activity in hepatitis C virus (HCV) infection. *Clin Exp Immunol* **109**, 451-457.

Cormier, E. G., Tsamis, F., Kajumo, F., Durso, R. J., Gardner, J. P. & Dragic, T. (2004). CD81 is an entry coreceptor for hepatitis C virus. *Proc Natl Acad Sci USA* **101**, 7270-7274. Epub 2004 May 7273.

Cosman, D., Fanger, N., Borges, L., Kubin, M., Chin, W., Peterson, L. & Hsu, M. L. (1997). A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. *Immunity* **7**, 273-282.

Cosman, D., Mullberg, J., Sutherland, C. L., Chin, W., Armitage, R., Fanslow, W., Kubin, M. & Chalupny, N. J. (2001). ULBPs, novel MHC class I-related molecules,

bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* **14**, 123-133.

Cramp, M. E., Rossol, S., Chokshi, S., Carucci, P., Williams, R. & Naoumov, N. V. (2000). Hepatitis C virus-specific T-cell reactivity during interferon and ribavirin treatment in chronic hepatitis C. *Gastroenterology* **118**, 346-355.

Cramp, M. E., Carucci, P., Rossol, S., Chokshi, S., Maertens, G., Williams, R. & Naoumov, N. V. (1999). Hepatitis C virus (HCV) specific immune responses in anti-HCV positive patients without hepatitis C viraemia. *Gut* **44**, 424-429.

Cribier, B., Schmitt, C., Kirn, A. & Stoll-Keller, F. (1998). Inhibition of hepatitis C virus adsorption to peripheral blood mononuclear cells by dextran sulfate. *Arch Virol* **143**, 375-379.

Crispe, I. N. (2003). Hepatic T cells and liver tolerance. *Nat Rev Immunol* **3**, 51-62.

Crotta, S., Stilla, A., Wack, A., D'Andrea, A., Nuti, S., D'Oro, U., Mosca, M., Filliponi, F., Brunetto, R. M., Bonino, F., Abrignani, S. & Valiante, N. M. (2002). Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. *Journal of Experimental Medicine* **195**, 35-41.

da Silva Correia, J., Soldau, K., Christen, U., Tobias, P. S. & Ulevitch, R. J. (2001). Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2. *J Biol Chem* **276**, 21129-21135. Epub 22001 Mar 21126.

Dahari, H., Feliu, A., Garcia-Retortillo, M., Forns, X. & Neumann, A. U. (2005). Second hepatitis C replication compartment indicated by viral dynamics during liver transplantation. *J Hepatol* **42**, 491-498. Epub 2005 Jan 2013.

Dammacco, F., Gatti, P. & Sansonno, D. (1998). Hepatitis C virus infection, mixed cryoglobulinemia, and non-Hodgkin's lymphoma: an emerging picture. *Leuk Lymphoma* **31**, 463-476.

Dardalhon, V., Jaleco, S., Rebouissou, C., Ferrand, C., Skander, N., Swainson, L., Tiberghien, P., Spits, H., Noraz, N. & Taylor, N. (2000). Highly efficient gene transfer in naive human T cells with a murine leukemia virus-based vector. *Blood* **96**, 885-893.

de Haas, C. J., van Leeuwen, H. J., Verhoef, J., van Kessel, K. P. & van Strijp, J. A. (2000). Analysis of lipopolysaccharide (LPS)-binding characteristics of serum components using gel filtration of FITC-labeled LPS. *J Immunol Methods* **242**, 79-89.

De Maria, A. & Moretta, L. (2000). HLA-class I-specific inhibitory receptors in HIV-1 infection. *Hum Immunol* **61**, 74-81.

De Maria, A., Fogli, M., Costa, P., Murdaca, G., Puppo, F., Mavilio, D., Moretta, A. & Moretta, L. (2003). The impaired NK cell cytolytic function in viremic HIV-1

infection is associated with a reduced surface expression of natural cytotoxicity receptors (NKp46, NKp30 and NKp44). *Eur J Immunol* **33**, 2410-2418.

Deignan, T., Curry, M. P., Doherty, D. G., Golden-Mason, L., Volkov, Y., Norris, S., Nolan, N., Traynor, O., McEntee, G., Hegarty, J. E. & O'Farrelly, C. (2002). Decrease in hepatic CD56(+) T cells and V alpha 24(+) natural killer T cells in chronic hepatitis C viral infection. *J Hepatol* **37**, 101-108.

Deleersnyder, V., Pillez, A., Wychowski, C., Blight, K., Xu, J., Hahn, Y. S., Rice, C. M. & Dubuisson, J. (1997). Formation of native hepatitis C virus glycoprotein complexes. *J Virol* **71**, 697-704.

Della Chiesa, M., Sivori, S., Castriconi, R., Marcenaro, E. & Moretta, A. (2005). Pathogen-induced private conversations between natural killer and dendritic cells. *Trends Microbiol* **13**, 128-136.

den Haan, J. M., Lehar, S. M. & Bevan, M. J. (2000). CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med* **192**, 1685-1696.

Deng, J., Dekruyff, R. H., Freeman, G. J., Umetsu, D. T. & Levy, S. (2002). Critical role of CD81 in cognate T-B cell interactions leading to Th2 responses. *Int Immunol* **14**, 513-523.

Deng, J., Yeung, V. P., Tsitoura, D., DeKruyff, R. H., Umetsu, D. T. & Levy, S. (2000). Allergen-induced airway hyperreactivity is diminished in CD81-deficient mice. *J Immunol* **165**, 5054-5061.

Denner, J., Persin, C., Vogel, T., Haustein, D., Norley, S. & Kurth, R. (1996). The immunosuppressive peptide of HIV-1 inhibits T and B lymphocyte stimulation. *J Acquir Immune Defic Syndr Hum Retrovirol* **12**, 442-450.

Diefenbach, A., Jensen, E. R., Jamieson, A. M. & Raulet, D. H. (2001). Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature* **413**, 165-171.

Diepolder, H. M., Zachoval, R., Hoffmann, R. M., Wierenga, E. A., Santantonio, T., Jung, M. C., Eichenlaub, D. & Pape, G. R. (1995). Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. *Lancet* **346**, 1006-1007.

Dinarello, C. A. & Fantuzzi, G. (2003). Interleukin-18 and host defense against infection. *J Infect Dis* **187**, S370-384.

Doherty, D. G., Norris, S., Madrigal-Estebas, L., McEntee, G., Traynor, O., Hegarty, J. E. & O'Farrelly, C. (1999). The human liver contains multiple populations of NK cells, T cells, and CD3+CD56+ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns. *J Immunol* **163**, 2314-2321.

Doherty, P. C. (1993). Immune exhaustion: driving virus-specific CD8+ T cells to death. *Trends Microbiol* **1**, 207-209.

Dolganiuc, A., Kodys, K., Kopasz, A., Marshall, C., Do, T., Romics, L., Jr., Mandrekar, P., Zapp, M. & Szabo, G. (2003). Hepatitis C virus core and nonstructural protein 3 proteins induce pro- and anti-inflammatory cytokines and inhibit dendritic cell differentiation. *J Immunol* **170**, 5615-5624.

Drummer, H. E., Wilson, K. A. & Poumbourios, P. (2002). Identification of the hepatitis C virus E2 glycoprotein binding site on the large extracellular loop of CD81. *J Virol* **76**, 11143-11147.

Drummer, H. E., Wilson, K. A. & Poumbourios, P. (2005). Determinants of CD81 dimerization and interaction with hepatitis C virus glycoprotein E2. *Biochem Biophys Res Commun* **328**, 251-257.

Dubuisson, J. (2000). Folding, assembly and subcellular localization of hepatitis C virus glycoproteins. *Curr Top Microbiol Immunol* **242**, 135-148.

Dubuisson, J. & Rice, C. M. (1996). Hepatitis C virus glycoprotein folding: disulfide bond formation and association with calnexin. *J Virol* **70**, 778-786.

Dubuisson, J., Penin, F. & Moradpour, D. (2002). Interaction of hepatitis C virus proteins with host cell membranes and lipids. *Trends Cell Biol* **12**, 517-523.

Dubuisson, J., Hsu, H. H., Cheung, R. C., Greenberg, H. B., Russell, D. G. & Rice, C. M. (1994). Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. *J Virol* **68**, 6147-6160.

Dubuisson, J., Duvet, S., Meunier, J. C., Op De Beeck, A., Cacan, R., Wychowski, C. & Cocquerel, L. (2000). Glycosylation of the hepatitis C virus envelope protein E1 is dependent on the presence of a downstream sequence on the viral polyprotein. *J Biol Chem* **275**, 30605-30609.

Ducoulombier, D., Roque-Afonso, A. M., Di Liberto, G., Penin, F., Kara, R., Richard, Y., Dussaix, E. & Feray, C. (2004). Frequent compartmentalization of hepatitis C virus variants in circulating B cells and monocytes. *Hepatology* **39**, 817-825.

Dunne, J., Lynch, S., O'Farrelly, C., Todryk, S., Hegarty, J. E., Feighery, C. & Doherty, D. G. (2001). Selective expansion and partial activation of human NK cells and NK receptor-positive T cells by IL-2 and IL-15. *J Immunol* **167**, 3129-3138.

Dykstra, M., Cherukuri, A. & Pierce, S. K. (2001). Rafts and synapses in the spatial organization of immune cell signaling receptors. *J Leukoc Biol* **70**, 699-707.

Dzionek, A., Fuchs, A., Schmidt, P., Cremer, S., Zysk, M., Miltenyi, S., Buck, D. W. & Schmitz, J. (2000). BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. *J Immunol* **165**, 6037-6046.

Dzionek, A., Sohma, Y., Nagafune, J., Cella, M., Colonna, M., Facchetti, F., Gunther, G., Johnston, I., Lanzavecchia, A., Nagasaka, T., Okada, T., Vermi, W., Winkels, G., Yamamoto, T., Zysk, M., Yamaguchi, Y. & Schmitz, J. (2001). BDCA-

2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. *J Exp Med* **194**, 1823-1834.

Egger, D., Wolk, B., Gosert, R., Bianchi, L., Blum, H. E., Moradpour, D. & Bienz, K. (2002). Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J Virol* **76**, 5974-5984.

Eisen-Vandervelde, A. L., Waggoner, S. N., Yao, Z. Q., Cale, E. M., Hahn, C. S. & Hahn, Y. S. (2004). Hepatitis C virus core selectively suppresses interleukin-12 synthesis in human macrophages by interfering with AP-1 activation. *J Biol Chem* **279**, 43479-43486. Epub 42004 Jul 43430.

Engel, P. & Tedder, T. F. (1994). New CD from the B cell section of the Fifth International Workshop on Human Leukocyte Differentiation Antigens. *Leuk Lymphoma* **13**, 61-64.

Engering, A. & Pieters, J. (2001). Association of distinct tetraspanins with MHC class II molecules at different subcellular locations in human immature dendritic cells. *Int Immunol* **13**, 127-134.

Engering, A., Van Vliet, S. J., Geijtenbeek, T. B. & Van Kooyk, Y. (2002). Subset of DC-SIGN(+) dendritic cells in human blood transmits HIV-1 to T lymphocytes. *Blood* **100**, 1780-1786.

Enomoto, N., Sakuma, I., Asahina, Y., Kurosaki, M., Murakami, T., Yamamoto, C., Izumi, N., Marumo, F. & Sato, C. (1995). Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. *J Clin Invest* **96**, 224-230.

Erickson, A. L., Kimura, Y., Igarashi, S., Eichelberger, J., Houghton, M., Sidney, J., McKinney, D., Sette, A., Hughes, A. L. & Walker, C. M. (2001). The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. *Immunity* **15**, 883-895.

Escola, J. M., Kleijmeer, M. J., Stoorvogel, W., Griffith, J. M., Yoshie, O. & Geuze, H. J. (1998). Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J Biol Chem* **273**, 20121-20127.

Esumi, M., Rikihisa, T., Nishimura, S., Goto, J., Mizuno, K., Zhou, Y. H. & Shikata, T. (1999). Experimental vaccine activities of recombinant E1 and E2 glycoproteins and hypervariable region 1 peptides of hepatitis C virus in chimpanzees. *Arch Virol* **144**, 973-980.

Exley, M. A., He, Q., Cheng, O., Wang, R. J., Cheney, C. P., Balk, S. P. & Koziel, M. J. (2002). Cutting edge: Compartmentalization of Th1-like noninvariant CD1d-reactive T cells in hepatitis C virus-infected liver. *J Immunol* **168**, 1519-1523.

Exley, M. A., Bigley, N. J., Cheng, O., Shaulov, A., Tahir, S. M., Carter, Q. L., Garcia, J., Wang, C., Patten, K., Stills, H. F., Alt, F. W., Snapper, S. B. & Balk, S. P. (2003). Innate immune response to encephalomyocarditis virus infection mediated by CD1d. *Immunology* **110**, 519-526.

Farci, P. & Purcell, R. H. (2000). Clinical significance of hepatitis C virus genotypes and quasispecies. *Semin Liver Dis* **20**, 103-126.

Farci, P., Alter, H. J., Wong, D. C., Miller, R. H., Govindarajan, S., Engle, R., Shapiro, M. & Purcell, R. H. (1994). Prevention of hepatitis C virus infection in chimpanzees after antibody-mediated in vitro neutralization. *Proc Natl Acad Sci USA* **91**, 7792-7796.

Farci, P., Shimoda, A., Wong, D., Cabezon, T., De Gioannis, D., Strazzera, A., Shimizu, Y., Shapiro, M., Alter, H. J. & Purcell, R. H. (1996). Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. *Proc Natl Acad Sci USA* **93**, 15394-15399.

Farci, P., Alter, H. J., Govindarajan, S., Wong, D. C., Engle, R., Lesniewski, R. R., Mushahwar, I. K., Desai, S. M., Miller, R. H., Ogata, N. & et al. (1992). Lack of protective immunity against reinfection with hepatitis C virus. *Science* **258**, 135-140.

Farci, P., Shimoda, A., Coiana, A., Diaz, G., Peddis, G., Melpolder, J. C., Strazzera, A., Chien, D. Y., Munoz, S. J., Balestrieri, A., Purcell, R. H. & Alter, H. J. (2000). The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. *Science* **288**, 339-344.

Farrell, H. E., Vally, H., Lynch, D. M., Fleming, P., Shellam, G. R., Scalzo, A. A. & Davis-Poynter, N. J. (1997). Inhibition of natural killer cells by a cytomegalovirus MHC class I homologue in vivo. *Nature* **386**, 510-514.

Fearon, D. T. & Carter, R. H. (1995). The CD19/CR2/TAPA-1 complex of B lymphocytes: linking natural to acquired immunity. *Annu Rev Immunol* **13**, 127-149.

Feigelson, S. W., Grabovsky, V., Shamri, R., Levy, S. & Alon, R. (2003). The CD81 tetraspanin facilitates instantaneous leukocyte VLA-4 adhesion strengthening to vascular cell adhesion molecule 1 (VCAM-1) under shear flow. *J Biol Chem* **278**, 51203-51212. Epub 52003 Oct 51207.

Feinstone, S. M., Alter, H. J., Dienes, H. P., Shimizu, Y., Popper, H., Blackmore, D., Sly, D., London, W. T. & Purcell, R. H. (1981). Non-A, non-B hepatitis in chimpanzees and marmosets. *J Infect Dis* **144**, 588-598.

Feray, C., Samuel, D., Thiers, V., Gigou, M., Pichon, F., Bismuth, A., Reynes, M., Maisonneuve, P., Bismuth, H. & Brechot, C. (1992). Reinfection of liver graft by hepatitis C virus after liver transplantation. *J Clin Invest* **89**, 1361-1365.

Ferlazzo, G., Tsang, M. L., Moretta, L., Melioli, G., Steinman, R. M. & Munz, C. (2002). Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. *J Exp Med* **195**, 343-351.

Fernandez, N. C., Lozier, A., Flament, C., Ricciardi-Castagnoli, P., Bellet, D., Suter, M., Perricaudet, M., Tursz, T., Maraskovsky, E. & Zitvogel, L. (1999). Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. *Nat Med* **5**, 405-411.

Ferri, C. & Zignego, A. L. (2000). Relation between infection and autoimmunity in mixed cryoglobulinemia. *Curr Opin Rheumatol* **12**, 53-60.

Ferri, C., Zignego, A. L. & Pileri, S. A. (2002). Cryoglobulins. *J Clin Pathol* **55**, 4-13.

Ferri, C., La Civita, L., Longombardo, G., Zignego, A. L. & Pasero, G. (1998). Mixed cryoglobulinaemia: a cross-road between autoimmune and lymphoproliferative disorders. *Lupus* **7**, 275-279.

Ferri, C., Greco, F., Longombardo, G., Palla, P., Moretti, A., Marzo, E., Mazzoni, A., Pasero, G., Bombardieri, S., Highfield, P. & et al. (1991). Association between hepatitis C virus and mixed cryoglobulinemia [see comment]. *Clin Exp Rheumatol* **9**, 621-624.

Ferri, C., Baicchi, U., la Civita, L., Greco, F., Longombardo, G., Mazzoni, A., Careccia, G., Bombardieri, S., Pasero, G., Zignego, A. L. & et al. (1993). Hepatitis C virus-related autoimmunity in patients with porphyria cutanea tarda. *Eur J Clin Invest* **23**, 851-855.

Ferri, C., Caracciolo, F., Zignego, A. L., La Civita, L., Monti, M., Longombardo, G., Lombardini, F., Greco, F., Capochiani, E., Mazzoni, A. & et al. (1994). Hepatitis C virus infection in patients with non-Hodgkin's lymphoma. *Br J Haematol* **88**, 392-394.

Flint, M. & McKeating, J. A. (1999). The C-terminal region of the hepatitis C virus E1 glycoprotein confers localization within the endoplasmic reticulum. *J Gen Virol* **80**, 1943-1947.

Flint, M. & McKeating, J. A. (2000). The role of the hepatitis C virus glycoproteins in infection. *Rev Med Virol* **10**, 101-117.

Flint, M., Logvinoff, C., Rice, C. M. & McKeating, J. A. (2004). Characterization of infectious retroviral pseudotype particles bearing hepatitis C virus glycoproteins. *J Virol* **78**, 6875-6882.

Flint, M., Thomas, J. M., Maidens, C. M., Shotton, C., Levy, S., Barclay, W. S. & McKeating, J. A. (1999a). Functional analysis of cell surface-expressed hepatitis C virus E2 glycoprotein. *J Virol* **73**, 6782-6790.

Flint, M., Dubuisson, J., Maidens, C., Harrop, R., Guile, G. R., Borrow, P. & McKeating, J. A. (2000). Functional characterization of intracellular and secreted forms of a truncated hepatitis C virus E2 glycoprotein. *J Virol* **74**, 702-709.

Flint, M., Maidens, C., Loomis-Price, L. D., Shotton, C., Dubuisson, J., Monk, P., Higginbottom, A., Levy, S. & McKeating, J. A. (1999b). Characterization of hepatitis

C virus E2 glycoprotein interaction with a putative cellular receptor, CD81. *J Virol* **73**, 6235-6244.

Fogler, W. E., Volker, K., McCormick, K. L., Watanabe, M., Ortaldo, J. R. & Wiltrout, R. H. (1996). NK cell infiltration into lung, liver, and subcutaneous B16 melanoma is mediated by VCAM-1/VLA-4 interaction. *J Immunol* **156**, 4707-4714.

Forns, X. & Bukh, J. (1999). The molecular biology of hepatitis C virus. Genotypes and quasispecies. *Clin Liver Dis* **3**, 693-716, vii.

Forns, X., Thimme, R., Govindarajan, S., Emerson, S. U., Purcell, R. H., Chisari, F. V. & Bukh, J. (2000). Hepatitis C virus lacking the hypervariable region 1 of the second envelope protein is infectious and causes acute resolving or persistent infection in chimpanzees. *Proc Natl Acad Sci USA* **97**, 13318-13323.

Foy, E., Li, K., Wang, C., Sumpter, R., Jr., Ikeda, M., Lemon, S. M. & Gale, M., Jr. (2003). Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science* **300**, 1145-1148. Epub 2003 Apr 1117.

Foy, E., Li, K., Sumpter, R., Jr., Loo, Y. M., Johnson, C. L., Wang, C., Fish, P. M., Yoneyama, M., Fujita, T., Lemon, S. M. & Gale, M., Jr. (2005). Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. *Proc Natl Acad Sci U S A* **102**, 2986-2991. Epub 2005 Feb 2914.

Frasca, L., Del Porto, P., Tuosto, L., Marinari, B., Scotta, C., Carbonari, M., Nicosia, A. & Piccolella, E. (1999). Hypervariable region 1 variants act as TCR antagonists for hepatitis C virus-specific CD4+ T cells. *J Immunol* **163**, 650-658.

French, A. R. & Yokoyama, W. M. (2003). Natural killer cells and viral infections. *Curr Opin Immunol* **15**, 45-51.

French, A. R., Pingel, J. T., Wagner, M., Bubic, I., Yang, L., Kim, S., Koszinowski, U., Jonjic, S. & Yokoyama, W. M. (2004). Escape of mutant double-stranded DNA virus from innate immune control. *Immunity* **20**, 747-756.

Frey, M., Packianathan, N. B., Fehniger, T. A., Ross, M. E., Wang, W. C., Stewart, C. C., Caligiuri, M. A. & Evans, S. S. (1998). Differential expression and function of L-selectin on CD56bright and CD56dim natural killer cell subsets. *J Immunol* **161**, 400-408.

Friberg, D. D., Bryant, J. L. & Whiteside, T. L. (1996). Measurements of Natural Killer (NK) Activity and NK-Cell Quantification. *Methods* **9**, 316-326.

Fritzsching, B., Schwer, B., Kartenbeck, J., Pedal, A., Horejsi, V. & Ott, M. (2002). Release and intercellular transfer of cell surface CD81 via microparticles. *J Immunol* **169**, 5531-5537.

Fruh, K., Ahn, K., Djaballah, H., Sempe, P., van Endert, P. M., Tampe, R., Peterson, P. A. & Yang, Y. (1995). A viral inhibitor of peptide transporters for antigen presentation. *Nature* **375**, 415-418.

Fuller, M. J., Khanolkar, A., Tebo, A. E. & Zajac, A. J. (2004). Maintenance, loss, and resurgence of T cell responses during acute, protracted, and chronic viral infections. *J Immunol* **172**, 4204-4214.

Gabrielli, A., Sambo, P., Zhang, Z. X., Candela, M., Savoldi, S., Manzin, A., Clementi, M., Amoroso, A., Sallberg, M. & Danieli, G. (1995). Humoral immune response and natural killer activity in patients with mixed cryoglobulinemia. *Clin Exp Rheumatol* **13**, S95-99.

Gahmberg, C. G. & Tolvanen, M. (1996). Why mammalian cell surface proteins are glycoproteins. *Trends Biochem Sci* **21**, 308-311.

Galandrini, R., Tassi, I., Mattia, G., Lenti, L., Piccoli, M., Frati, L. & Santoni, A. (2002). SH2-containing inositol phosphatase (SHIP-1) transiently translocates to raft domains and modulates CD16-mediated cytotoxicity in human NK cells. *Blood* **100**, 4581-4589. Epub 2002 Aug 4508.

Galatiuc, C., Gherman, M., Metes, D., Sulica, A., DeLeo, A., Whiteside, T. L. & Herberman, R. B. (1995). Natural killer (NK) activity in human responders and nonresponders to stimulation by anti-CD16 antibodies. *Cell Immunol* **163**, 167-177.

Gale, M., Jr., Blakely, C. M., Kwieciszewski, B., Tan, S. L., Dossett, M., Tang, N. M., Korth, M. J., Polyak, S. J., Gretch, D. R. & Katze, M. G. (1998). Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. *Mol Cell Biol* **18**, 5208-5218.

Gale, M. J., Jr., Korth, M. J., Tang, N. M., Tan, S. L., Hopkins, D. A., Dever, T. E., Polyak, S. J., Gretch, D. R. & Katze, M. G. (1997). Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology* **230**, 217-227.

Gao, L., Aizaki, H., He, J. W. & Lai, M. M. (2004). Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. *J Virol* **78**, 3480-3488.

Gardner, J. P., Durso, R. J., Arrigale, R. R., Donovan, G. P., Maddon, P. J., Dragic, T. & Olson, W. C. (2003). L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. *Proc Natl Acad Sci USA* **100**, 4498-4503. Epub 2003 Apr 4403.

Garson, J. A., Lubach, D., Passas, J., Whitby, K. & Grant, P. R. (1999). Suramin blocks hepatitis C binding to human hepatoma cells in vitro. *J Med Virol* **57**, 238-242.

Geijtenbeek, T. B., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Adema, G. J., van Kooyk, Y. & Figdor, C. G. (2000a). Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* **100**, 575-585.

Geijtenbeek, T. B., Van Vliet, S. J., Koppel, E. A., Sanchez-Hernandez, M., Vandenbroucke-Grauls, C. M., Appelmelk, B. & Van Kooyk, Y. (2003). Mycobacteria target DC-SIGN to suppress dendritic cell function. *J Exp Med* **197**, 7-17.

Geijtenbeek, T. B., Krooshoop, D. J., Bleijs, D. A., van Vliet, S. J., van Duijnhoven, G. C., Grabovsky, V., Alon, R., Figdor, C. G. & van Kooyk, Y. (2000b). DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking. *Nat Immunol* **1**, 353-357.

Geijtenbeek, T. B., Kwon, D. S., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Middel, J., Cornelissen, I. L., Nottet, H. S., KewalRamani, V. N., Littman, D. R., Figdor, C. G. & van Kooyk, Y. (2000c). DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* **100**, 587-597.

Geleziunas, R., Xu, W., Takeda, K., Ichijo, H. & Greene, W. C. (2001). HIV-1 Nef inhibits ASK1-dependent death signalling providing a potential mechanism for protecting the infected host cell. *Nature* **410**, 834-838.

Gerlach, J. T., Diepolder, H. M., Jung, M. C., Gruener, N. H., Schraut, W. W., Zachoval, R., Hoffmann, R., Schirren, C. A., Santantonio, T. & Pape, G. R. (1999). Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. *Gastroenterology* **117**, 933-941.

Gerlach, J. T., Diepolder, H. M., Zachoval, R., Gruener, N. H., Jung, M. C., Ulsenheimer, A., Schraut, W. W., Schirren, C. A., Waechtler, M., Backmund, M. & Pape, G. R. (2003). Acute hepatitis C: high rate of both spontaneous and treatment-induced viral clearance. *Gastroenterology* **125**, 80-88.

Gerosa, F., Baldani-Guerra, B., Nisii, C., Marchesini, V., Carra, G. & Trinchieri, G. (2002). Reciprocal activating interaction between natural killer cells and dendritic cells. *J Exp Med* **195**, 327-333.

Gerotto, M., Dal Pero, F., Loffreda, S., Bianchi, F. B., Alberti, A. & Lenzi, M. (2001). A 385 insertion in the hypervariable region 1 of hepatitis C virus E2 envelope protein is found in some patients with mixed cryoglobulinemia type 2. *Blood* **98**, 2657-2663.

Gerotto, M., Dal Pero, F., Sullivan, D. G., Chemello, L., Cavalletto, L., Polyak, S. J., Pontisso, P., Gretch, D. R. & Alberti, A. (1999). Evidence for sequence selection within the non-structural 5A gene of hepatitis C virus type 1b during unsuccessful treatment with interferon-alpha. *J Viral Hepat* **6**, 367-372.

Gherman, M., Manciulea, M., Bancu, A. C., Sulica, A., Stanworth, D. R. & Herberman, R. B. (1987). Regulation of human natural cytotoxicity by IgG--I. Characterization of the structural site on monomeric IgG responsible for inhibiting natural killer cell activity. *Mol Immunol* **24**, 743-750.

Gisbert, J. P., Garcia-Buey, L., Pajares, J. M. & Moreno-Otero, R. (2003). Prevalence of hepatitis C virus infection in porphyria cutanea tarda: systematic review and meta-analysis. *J Hepatol* **39**, 620-627.

Goffard, A. & Dubuisson, J. (2003). Glycosylation of hepatitis C virus envelope proteins. *Biochimie* **85**, 295-301.

Gong, G. Z., Lai, L. Y., Jiang, Y. F., He, Y. & Su, X. S. (2003). HCV replication in PBMC and its influence on interferon therapy. *World J Gastroenterol* **9**, 291-294.

Goulder, P. J. & Watkins, D. I. (2004). HIV and SIV CTL escape: implications for vaccine design. *Nat Rev Immunol* **4**, 630-640.

Goutagny, N., Fatmi, A., De Ledinghen, V., Penin, F., Couzigou, P., Inchauspe, G. & Bain, C. (2003). Evidence of viral replication in circulating dendritic cells during hepatitis C virus infection. *J Infect Dis* **187**, 1951-1958. Epub 2003 May 1929.

Graf, G. A., Connell, P. M., van der Westhuyzen, D. R. & Smart, E. J. (1999). The class B, type I scavenger receptor promotes the selective uptake of high density lipoprotein cholesterol ethers into caveolae. *J Biol Chem* **274**, 12043-12048.

Grakoui, A., McCourt, D. W., Wychowski, C., Feinstone, S. M. & Rice, C. M. (1993). A second hepatitis C virus-encoded proteinase. *Proc Natl Acad Sci USA* **90**, 10583-10587.

Griffin, S. D., Beales, L. P., Clarke, D. S., Worsfold, O., Evans, S. D., Jaeger, J., Harris, M. P. & Rowlands, D. J. (2003). The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. *FEBS Lett* **535**, 34-38.

Grubor-Bauk, B., Simmons, A., Mayrhofer, G. & Speck, P. G. (2003). Impaired clearance of herpes simplex virus type 1 from mice lacking CD1d or NKT cells expressing the semivariant V alpha 14-J alpha 281 TCR. *J Immunol* **170**, 1430-1434.

Gruener, N. H., Lechner, F., Jung, M. C., Diepolder, H., Gerlach, T., Lauer, G., Walker, B., Sullivan, J., Phillips, R., Pape, G. R. & Klenerman, P. (2001). Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *J Virol* **75**, 5550-5558.

Gruner, N. H., Gerlach, T. J., Jung, M. C., Diepolder, H. M., Schirren, C. A., Schraut, W. W., Hoffmann, R., Zachoval, R., Santantonio, T., Cucchiarini, M., Cerny, A. & Pape, G. R. (2000). Association of hepatitis C virus-specific CD8+ T cells with viral clearance in acute hepatitis C. *J Infect Dis* **181**, 1528-1536. Epub 2000 May 1525.

Guma, M., Angulo, A., Vilches, C., Gomez-Lozano, N., Malats, N. & Lopez-Botet, M. (2004). Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood* **104**, 3664-3671. Epub 2004 Aug 3610.

Habersetzer, F., Fournillier, A., Dubuisson, J., Rosa, D., Abrignani, S., Wychowski, C., Nakano, I., Trepo, C., Desgranges, C. & Inchauspe, G. (1998). Characterization of human monoclonal antibodies specific to the hepatitis C virus glycoprotein E2 with in vitro binding neutralization properties. *Virology* **249**, 32-41.

Hadlock, K. G., Lanford, R. E., Perkins, S., Rowe, J., Yang, Q., Levy, S., Pileri, P., Abrignani, S. & Foung, S. K. (2000). Human monoclonal antibodies that inhibit binding of hepatitis C virus E2 protein to CD81 and recognize conserved conformational epitopes. *J Virol* **74**, 10407-10416.

Hadziyannis, S. J. & Koskinas, J. S. (2004). Differences in epidemiology, liver disease and treatment response among HCV genotypes. *Hepatol Res* **29**, 129-135.

Hahn, C. S., Cho, Y. G., Kang, B. S., Lester, I. M. & Hahn, Y. S. (2000). The HCV core protein acts as a positive regulator of fas-mediated apoptosis in a human lymphoblastoid T cell line. *Virology* **276**, 127-137.

Halary, F., Amara, A., Lortat-Jacob, H., Messerle, M., Delaunay, T., Houles, C., Fieschi, F., Arenzana-Seisdedos, F., Moreau, J. F. & Dechanet-Merville, J. (2002). Human cytomegalovirus binding to DC-SIGN is required for dendritic cell infection and target cell trans-infection. *Immunity* **17**, 653-664.

Hamaia, S., Li, C. & Allain, J. P. (2001). The dynamics of hepatitis C virus binding to platelets and 2 mononuclear cell lines. *Blood* **98**, 2293-2300.

Harcourt, G., Hellier, S., Bunce, M., Satsangi, J., Collier, J., Chapman, R., Phillips, R. & Klenerman, P. (2001). Effect of HLA class II genotype on T helper lymphocyte responses and viral control in hepatitis C virus infection. *J Viral Hepat* **8**, 174-179.

Hardy, R. W., Marcotrigiano, J., Blight, K. J., Majors, J. E. & Rice, C. M. (2003). Hepatitis C virus RNA synthesis in a cell-free system isolated from replicon-containing hepatoma cells. *J Virol* **77**, 2029-2037.

Haziot, A., Ferrero, E., Kontgen, F., Hijiya, N., Yamamoto, S., Silver, J., Stewart, C. L. & Goyert, S. M. (1996). Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. *Immunity* **4**, 407-414.

He, X. S., Rehermann, B., Lopez-Labrador, F. X., Boisvert, J., Cheung, R., Mumm, J., Wedemeyer, H., Berenguer, M., Wright, T. L., Davis, M. M. & Greenberg, H. B. (1999). Quantitative analysis of hepatitis C virus-specific CD8(+) T cells in peripheral blood and liver using peptide-MHC tetramers. *Proc Natl Acad Sci USA* **96**, 5692-5697.

Heile, J. M., Fong, Y. L., Rosa, D., Berger, K., Saletti, G., Campagnoli, S., Bensi, G., Capo, S., Coates, S., Crawford, K., Dong, C., Wininger, M., Baker, C., Cousens, L., Chien, D., Ng, P., Archangel, P., Grandi, G., Houghton, M. & Abrignani, S. (2000). Evaluation of hepatitis C virus glycoprotein E2 for vaccine design: an endoplasmic reticulum-retained recombinant protein is superior to secreted recombinant protein and DNA-based vaccine candidates. *Journal of Virology* **74**, 6885-6892.

Heller, T., Saito, S., Auerbach, J., Williams, T., Moreen, T. R., Jazwinski, A., Cruz, B., Jeurkar, N., Sapp, R., Luo, G. & Liang, T. J. (2005). An in vitro model of hepatitis C virion production. *Proc Natl Acad Sci USA* **102**, 2579-2583. Epub 2005 Feb 2578.

Hemler, M. E. (2001). Specific tetraspanin functions. *J Cell Biol* **155**, 1103-1107. Epub 2001 Dec 1124.

Henkart, P. A. (1994). Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *Immunity* **1**, 343-346.

Hermans, I. F., Ritchie, D. S., Daish, A., Yang, J., Kehry, M. R. & Ronchese, F. (1999). Impaired ability of MHC class II-/- dendritic cells to provide tumor protection is rescued by CD40 ligation. *J Immunol* **163**, 77-81.

Herzer, K., Falk, C. S., Encke, J., Eichhorst, S. T., Ulsenheimer, A., Seliger, B. & Krammer, P. H. (2003). Upregulation of major histocompatibility complex class I on liver cells by hepatitis C virus core protein via p53 and TAP1 impairs natural killer cell cytotoxicity. *J Virol* **77**, 8299-8309.

Hiasa, Y., Horiike, N., Akbar, S. M., Saito, I., Miyamura, T., Matsuura, Y. & Onji, M. (1998). Low stimulatory capacity of lymphoid dendritic cells expressing hepatitis C virus genes. *Biochem Biophys Res Commun* **249**, 90-95.

Higginbottom, A., Quinn, E. R., Kuo, C. C., Flint, M., Wilson, L. H., Bianchi, E., Nicosia, A., Monk, P. N., McKeating, J. A. & Levy, S. (2000). Identification of amino acid residues in CD81 critical for interaction with hepatitis C virus envelope glycoprotein E2. *J Virol* **74**, 3642-3649.

Hiroishi, K., Kita, H., Kojima, M., Okamoto, H., Moriyama, T., Kaneko, T., Ishikawa, T., Ohnishi, S., Aikawa, T., Tanaka, N., Yazaki, Y., Mitamura, K. & Imawari, M. (1997). Cytotoxic T lymphocyte response and viral load in hepatitis C virus infection. *Hepatology* **25**, 705-712.

Hofmann, W. P., Zeuzem, S. & Sarrazin, C. (2005). Hepatitis C virus-related resistance mechanisms to interferon alpha-based antiviral therapy. *J Clin Virol* **32**, 86-91.

Hofmann, W. P., Herrmann, E., Kronenberger, B., Merkwirth, C., Welsch, C., Lengauer, T., Zeuzem, S. & Sarrazin, C. (2004). Association of HCV-related mixed cryoglobulinemia with specific mutational pattern of the HCV E2 protein and CD81 expression on peripheral B lymphocytes. *Blood* **104**, 1228-1229.

Hong, Z., Beaudet-Miller, M., Lanford, R. E., Guerra, B., Wright-Minogue, J., Skelton, A., Baroudy, B. M., Reyes, G. R. & Lau, J. Y. (1999). Generation of transmissible hepatitis C virions from a molecular clone in chimpanzees. *Virology* **256**, 36-44.

Hoofnagle, J. H. (1997). Hepatitis C: the clinical spectrum of disease. *Hepatology* **26**, 15S-20S.

Hoofnagle, J. H. (2002). Course and outcome of hepatitis C. *Hepatology* **36**, S21-29.

Hoofnagle, J. H., Carithers, R. L., Jr., Shapiro, C. & Ascher, N. (1995). Fulminant hepatic failure: summary of a workshop. *Hepatology* **21**, 240-252.

Hope, R. G. & McLauchlan, J. (2000). Sequence motifs required for lipid droplet association and protein stability are unique to the hepatitis C virus core protein. *J Gen Virol* **81**, 1913-1925.

Hsu, M., Zhang, J., Flint, M., Logvinoff, C., Cheng-Mayer, C., Rice, C. M. & McKeating, J. A. (2003). Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 7271-7276.

Hu, Y. W., Rocheleau, L., Larke, B., Chui, L., Lee, B., Ma, M., Liu, S., Omlin, T., Pelchat, M. & Brown, E. G. (2005). Immunoglobulin mimicry by Hepatitis C Virus envelope protein E2. *Virology* **332**, 538-549.

Husemann, J. & Silverstein, S. C. (2001). Expression of scavenger receptor class B, type I, by astrocytes and vascular smooth muscle cells in normal adult mouse and human brain and in Alzheimer's disease brain. *Am J Pathol* **158**, 825-832.

Hussy, P., Langen, H., Mous, J. & Jacobsen, H. (1996). Hepatitis C virus core protein: carboxy-terminal boundaries of two processed species suggest cleavage by a signal peptide peptidase. *Virology* **224**, 93-104.

Imachi, H., Murao, K., Hiramine, C., Sayo, Y., Sato, M., Hosokawa, H., Ishida, T., Kodama, T., Quehenberger, O., Steinberg, D. & Takahara, J. (2000). Human scavenger receptor B1 is involved in recognition of apoptotic thymocytes by thymic nurse cells. *Lab Invest* **80**, 263-270.

Imai, T. & Yoshie, O. (1993). C33 Antigen and M38 Antigen Recognized by Monoclonal-Antibodies Inhibitory to Syncytium Formation by Human T-Cell Leukemia- Virus Type-1 Are Both Members of the Transmembrane-4 Superfamily and Associate with Each Other and with Cd4 or Cd8 in T-Cells. *Journal of Immunology* **151**, 6470-6481.

Ito, T., Amakawa, R., Inaba, M., Ikehara, S., Inaba, K. & Fukuhara, S. (2001). Differential regulation of human blood dendritic cell subsets by IFNs. *J Immunol* **166**, 2961-2969.

Jacobs, R., Heiken, H. & Schmidt, R. E. (2005). Mutual interference of HIV and natural killer cell-mediated immune response. *Mol Immunol* **42**, 239-249.

Jacobs, R., Hintzen, G., Kemper, A., Beul, K., Kempf, S., Behrens, G., Sykora, K. W. & Schmidt, R. E. (2001). CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. *Eur J Immunol* **31**, 3121-3127.

Janssen, E. M., Lemmens, E. E., Wolfe, T., Christen, U., von Herrath, M. G. & Schoenberger, S. P. (2003). CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* **421**, 852-856. Epub 2003 Feb 2009.

Janssen, E. M., Droin, N. M., Lemmens, E. E., Pinkoski, M. J., Bensinger, S. J., Ehst, B. D., Griffith, T. S., Green, D. R. & Schoenberger, S. P. (2005). CD4+ T-cell

help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature* **434**, 88-93.

Jinushi, M., Takehara, T., Tatsumi, T., Kanto, T., Groh, V., Spies, T., Suzuki, T., Miyagi, T. & Hayashi, N. (2003a). Autocrine/paracrine IL-15 that is required for type I IFN-mediated dendritic cell expression of MHC class I-related chain A and B is impaired in hepatitis C virus infection. *J Immunol* **171**, 5423-5429.

Jinushi, M., Takehara, T., Tatsumi, T., Kanto, T., Miyagi, T., Suzuki, T., Kanazawa, Y., Hiramatsu, N. & Hayashi, N. (2004). Negative regulation of NK cell activities by inhibitory receptor CD94/NKG2A leads to altered NK cell-induced modulation of dendritic cell functions in chronic hepatitis C virus infection. *J Immunol* **173**, 6072-6081.

Jinushi, M., Takehara, T., Kanto, T., Tatsumi, T., Groh, V., Spies, T., Miyagi, T., Suzuki, T., Sasaki, Y. & Hayashi, N. (2003b). Critical role of MHC class I-related chain A and B expression on IFN-alpha-stimulated dendritic cells in NK cell activation: impairment in chronic hepatitis C virus infection. *J Immunol* **170**, 1249-1256.

Johnson, T. R., Hong, S., Van Kaer, L., Koezuka, Y. & Graham, B. S. (2002). NK T cells contribute to expansion of CD8(+) T cells and amplification of antiviral immune responses to respiratory syncytial virus. *J Virol* **76**, 4294-4303.

Joseph, B. S., Lampert, P. W. & Oldstone, M. B. (1975). Replication and persistence of measles virus in defined subpopulations of human leukocytes. *J Virol* **16**, 1638-1649.

Kadowaki, N., Antonenko, S., Lau, J. Y. & Liu, Y. J. (2000). Natural interferon alpha/beta-producing cells link innate and adaptive immunity. *J Exp Med* **192**, 219-226.

Kaimori, A., Kanto, T., Kwang Limn, C., Komoda, Y., Oki, C., Inoue, M., Miyatake, H., Itose, I., Sakakibara, M., Yakushijin, T., Takehara, T., Matsuura, Y. & Hayashi, N. (2004). Pseudotype hepatitis C virus enters immature myeloid dendritic cells through the interaction with lectin. *Virology* **324**, 74-83.

Kalams, S. A. & Walker, B. D. (1998). The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. *J Exp Med* **188**, 2199-2204.

Kambayashi, T., Assarsson, E., Michaelsson, J., Berglund, P., Diehl, A. D., Chambers, B. J. & Ljunggren, H. G. (2000). Emergence of CD8+ T cells expressing NK cell receptors in influenza A virus-infected mice. *J Immunol* **165**, 4964-4969.

Kanto, T., Hayashi, N., Takehara, T., Tatsumi, T., Kuzushita, N., Ito, A., Sasaki, Y., Kasahara, A. & Hori, M. (1999). Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. *J Immunol* **162**, 5584-5591.

Kanto, T., Inoue, M., Miyatake, H., Sato, A., Sakakibara, M., Yakushijin, T., Oki, C., Itose, I., Hiramatsu, N., Takehara, T., Kasahara, A. & Hayashi, N. (2004). Reduced numbers and impaired ability of myeloid and plasmacytoid dendritic cells to

polarize T helper cells in chronic hepatitis C virus infection. *J Infect Dis* **190**, 1919-1926. Epub 2004 Oct 1927.

Kato, N. (2001). Molecular virology of hepatitis C virus. *Acta Med Okayama* **55**, 133-159.

Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T. & Shimotohno, K. (1990). Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc Natl Acad Sci USA* **87**, 9524-9528.

Kato, N., Ootsuyama, Y., Sekiya, H., Ohkoshi, S., Nakazawa, T., Hijikata, M. & Shimotohno, K. (1994). Genetic drift in hypervariable region 1 of the viral genome in persistent hepatitis C virus infection. *J Virol* **68**, 4776-4784.

Katze, M. G., He, Y. & Gale, M., Jr. (2002). Viruses and interferon: a fight for supremacy. *Nat Rev Immunol* **2**, 675-687.

Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Motoki, K., Ueno, H., Nakagawa, R., Sato, H., Kondo, E., Koseki, H. & Taniguchi, M. (1997). CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* **278**, 1626-1629.

Kawarabayashi, N., Seki, S., Hatsuse, K., Ohkawa, T., Koike, Y., Aihara, T., Habu, Y., Nakagawa, R., Ami, K., Hiraide, H. & Mochizuki, H. (2000). Decrease of CD56(+)T cells and natural killer cells in cirrhotic livers with hepatitis C may be involved in their susceptibility to hepatocellular carcinoma. *Hepatology* **32**, 962-969.

Keck, Z. Y., Op De Beeck, A., Hadlock, K. G., Xia, J., Li, T. K., Dubuisson, J. & Foung, S. K. (2004). Hepatitis C virus e2 has three immunogenic domains containing conformational epitopes with distinct properties and biological functions. *J Virol* **78**, 9224-9232.

Kelic, S., Levy, S., Suarez, C. & Weinstein, D. E. (2001). CD81 regulates neuron-induced astrocyte cell-cycle exit. *Mol Cell Neurosci* **17**, 551-560.

Kelleher, M. & Beverley, P. C. (2001). Lipopolysaccharide modulation of dendritic cells is insufficient to mature dendritic cells to generate CTLs from naive polyclonal CD8+ T cells in vitro, whereas CD40 ligation is essential. *J Immunol* **167**, 6247-6255.

Khakoo, S. I., Thio, C. L., Martin, M. P., Brooks, C. R., Gao, X., Astemborski, J., Cheng, J., Goedert, J. J., Vlahov, D., Hilgartner, M., Cox, S., Little, A. M., Alexander, G. J., Cramp, M. E., O'Brien, S. J., Rosenberg, W. M., Thomas, D. L. & Carrington, M. (2004). HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science* **305**, 872-874.

Khorsi, H., Castelain, S., Wyseur, A., Izopet, J., Canva, V., Rombout, A., Capron, D., Capron, J. P., Lunel, F., Stuyver, L. & Duverlie, G. (1997). Mutations of hepatitis C virus 1b NS5A 2209-2248 amino acid sequence do not predict the response to recombinant interferon-alfa therapy in French patients. *J Hepatol* **27**, 72-77.

Kim, H. S., Lee, J. K., Yang, I. H., Ahn, J. K., Oh, Y. I., Kim, C. J., Kim, Y. S. & Lee, C. K. (2002). Identification of hepatitis C virus core domain inducing suppression of allostimulatory capacity of dendritic cells. *Arch Pharm Res* **25**, 364-369.

Kitadokoro, K., Bordo, D., Galli, G., Petracca, R., Falugi, F., Abrignani, S., Grandi, G. & Bolognesi, M. (2001). CD81 extracellular domain 3D structure: insight into the tetraspanin superfamily structural motifs. *Embo Journal* **20**, 12-18.

Kittlesen, D. J., Chianese-Bullock, K. A., Yao, Z. Q., Braciale, T. J. & Hahn, Y. S. (2000). Interaction between complement receptor gC1qR and hepatitis C virus core protein inhibits T-lymphocyte proliferation. *J Clin Invest* **106**, 1239-1249.

Kjellen, L. & Lindahl, U. (1991). Proteoglycans: structures and interactions. *Annu Rev Biochem* **60**, 443-475.

Klenerman, P., Lechner, F., Kantzanou, M., Ciurea, A., Hengartner, H. & Zinkernagel, R. (2000). Viral escape and the failure of cellular immune responses. *Science* **289**, 2003.

Kloover, J. S., Grauls, G. E., Blok, M. J., Vink, C. & Bruggeman, C. A. (2002). A rat cytomegalovirus strain with a disruption of the r144 MHC class I-like gene is attenuated in the acute phase of infection in neonatal rats. *Arch Virol* **147**, 813-824.

Knolle, P. A. & Gerken, G. (2000). Local control of the immune response in the liver. *Immunol Rev* **174**, 21-34.

Kolesnikova, T. V., Stipp, C. S., Rao, R. M., Lane, W. S., Luscinskas, F. W. & Hemler, M. E. (2004). EWI-2 modulates lymphocyte integrin alpha4beta1 functions. *Blood* **103**, 3013-3019. Epub 2003 Dec 3018.

Korenaga, M., Hino, K., Katoh, Y., Yamaguchi, Y., Okuda, M., Yoshioka, K. & Okita, K. (2001). A possible role of hypervariable region 1 quasispecies in escape of hepatitis C virus particles from neutralization. *J Viral Hepat* **8**, 331-340.

Koziel, M. J., Dudley, D., Wong, J. T., Dienstag, J., Houghton, M., Ralston, R. & Walker, B. D. (1992). Intrahepatic cytotoxic T lymphocytes specific for hepatitis C virus in persons with chronic hepatitis. *J Immunol* **149**, 3339-3344.

Koziel, M. J., Dudley, D., Afdhal, N., Choo, Q. L., Houghton, M., Ralston, R. & Walker, B. D. (1993). Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes recognize epitopes in the core and envelope proteins of HCV. *J Virol* **67**, 7522-7532.

Koziel, M. J., Dudley, D., Afdhal, N., Grakoui, A., Rice, C. M., Choo, Q. L., Houghton, M. & Walker, B. D. (1995). HLA class I-restricted cytotoxic T lymphocytes specific for hepatitis C virus. Identification of multiple epitopes and characterization of patterns of cytokine release. *J Clin Invest* **96**, 2311-2321.

Krieger, M. (1999). Charting the fate of the "good cholesterol": identification and characterization of the high-density lipoprotein receptor SR-BI. *Annu Rev Biochem* **68**, 523-558.

Krieger, M. (2001). Scavenger receptor class B type I is a multiligand HDL receptor that influences diverse physiologic systems. *J Clin Invest* **108**, 793-797.

Kronenberger, B., Ruster, B., Elez, R., Weber, S., Piiper, A., Lee, J. H., Roth, W. K. & Zeuzem, S. (2001). Interferon alfa down-regulates CD81 in patients with chronic hepatitis C. *Hepatology* **33**, 1518-1526.

Kronenberger, B., Sarrazin, C., Hofmann, W. P., von Wagner, M., Herrmann, E., Welsch, C., Elez, R., Ruster, B., Piiper, A. & Zeuzem, S. (2004). Mutations in the putative HCV-E2 CD81 binding regions and correlation with cell surface CD81 expression. *J Viral Hepat* **11**, 310-318.

Kubota, A., Kubota, S., Farrell, H. E., Davis-Poynter, N. & Takei, F. (1999). Inhibition of NK cells by murine CMV-encoded class I MHC homologue m144. *Cell Immunol* **191**, 145-151.

Kuo, G., Choo, Q. L., Alter, H. J., Gitnick, G. L., Redeker, A. G., Purcell, R. H., Miyamura, T., Dienstag, J. L., Alter, M. J., Stevens, C. E., Tegtmeier, G. E., Bonino, F., Colombo, M., Lee, W. S., Kuo, C., Berger, K., Shuster, J. R., Overby, L. R., Bradley, D. W. & Houghton, M. (1989). An Assay for Circulating Antibodies to a Major Etiologic Virus of Human Non-a, Non-B-Hepatitis. *Science* **244**, 362-364.

Kwon, D. S., Gregorio, G., Bitton, N., Hendrickson, W. A. & Littman, D. R. (2002). DC-SIGN-mediated internalization of HIV is required for trans-enhancement of T cell infection. *Immunity* **16**, 135-144.

Lagaudriere-Gesbert, C., Le Naour, F., Lebel-Binay, S., Billard, M., Lemichez, E., Boquet, P., Boucheix, C., Conjeaud, H. & Rubinstein, E. (1997). Functional analysis of four tetraspans, CD9, CD53, CD81, and CD82, suggests a common role in costimulation, cell adhesion, and migration: only CD9 upregulates HB-EGF activity. *Cell Immunol* **182**, 105-112.

Lambot, M., Fretier, S., Op De Beeck, A., Quatannens, B., Lestavel, S., Clavey, V. & Dubuisson, J. (2002). Reconstitution of hepatitis C virus envelope glycoproteins into liposomes as a surrogate model to study virus attachment. *J Biol Chem* **277**, 20625-20630. Epub 22002 Apr 20625.

Lan, K. H., Sheu, M. L., Hwang, S. J., Yen, S. H., Chen, S. Y., Wu, J. C., Wang, Y. J., Kato, N., Omata, M., Chang, F. Y. & Lee, S. D. (2002). HCV NS5A interacts with p53 and inhibits p53-mediated apoptosis. *Oncogene* **21**, 4801-4811.

Lanford, R. E., Bigger, C., Bassett, S. & Klimpel, G. (2001). The chimpanzee model of hepatitis C virus infections. *Ilar J* **42**, 117-126.

Lanier, L. L. (1998). NK cell receptors. *Annu Rev Immunol* **16**, 359-393.

Lanier, L. L. (2003). Natural killer cell receptor signaling. *Curr Opin Immunol* **15**, 308-314.

Lanier, L. L. (2005). NK cell recognition. *Annu Rev Immunol* **23**, 225-274.

Lanier, L. L., Corliss, B., Wu, J. & Phillips, J. H. (1998). Association of DAP12 with activating CD94/NKG2C NK cell receptors. *Immunity* **8**, 693-701.

Large, M. K., Kittlesen, D. J. & Hahn, Y. S. (1999). Suppression of host immune response by the core protein of hepatitis C virus: possible implications for hepatitis C virus persistence. *J Immunol* **162**, 931-938.

Laskus, T., Radkowski, M., Wang, L. F., Nowicki, M. & Rakela, J. (2000). Uneven distribution of hepatitis C virus quasispecies in tissues from subjects with end-stage liver disease: confounding effect of viral adsorption and mounting evidence for the presence of low-level extrahepatic replication. *J Virol* **74**, 1014-1017.

Lavillette, D., Tarr, A. W., Voisset, C., Donot, P., Bartosch, B., Bain, C., Patel, A. H., Dubuisson, J., Ball, J. K. & Cosset, F. L. (2005a). Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus. *Hepatology* **41**, 265-274.

Lavillette, D., Morice, Y., Germanidis, G., Donot, P., Soulier, A., Pagkalos, E., Sakellariou, G., Intrator, L., Bartosch, B., Pawlotsky, J. M. & Cosset, F. L. (2005b). Human serum facilitates hepatitis C virus infection, and neutralizing responses inversely correlate with viral replication kinetics at the acute phase of hepatitis C virus infection. *J Virol* **79**, 6023-6034.

Le Bon, A. & Tough, D. F. (2002). Links between innate and adaptive immunity via type I interferon. *Curr Opin Immunol* **14**, 432-436.

Le Bon, A., Etchart, N., Rossmann, C., Ashton, M., Hou, S., Gewert, D., Borrow, P. & Tough, D. F. (2003). Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. *Nat Immunol* **4**, 1009-1015. Epub 2003 Sep 1021.

Lebel-Binay, S., Lagaudriere, C., Fradelizi, D. & Conjeaud, H. (1995). CD82, member of the tetra-span-transmembrane protein family, is a costimulatory protein for T cell activation. *J Immunol* **155**, 101-110.

Lechner, F., Wong, D. K., Dunbar, P. R., Chapman, R., Chung, R. T., Dohrenwend, P., Robbins, G., Phillips, R., Klenerman, P. & Walker, B. D. (2000a). Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med* **191**, 1499-1512.

Lechner, F., Gruener, N. H., Urbani, S., Uggeri, J., Santantonio, T., Kammer, A. R., Cerny, A., Phillips, R., Ferrari, C., Pape, G. R. & Klenerman, P. (2000b). CD8+ T lymphocyte responses are induced during acute hepatitis C virus infection but are not sustained. *Eur J Immunol* **30**, 2479-2487.

Lee, C. H., Choi, Y. H., Yang, S. H., Lee, C. W., Ha, S. J. & Sung, Y. C. (2001). Hepatitis C virus core protein inhibits interleukin 12 and nitric oxide production from activated macrophages. *Virology* **279**, 271-279.

Lehmann, M., Meyer, M. F., Monazahian, M., Tillmann, H. L., Manns, M. P. & Wedemeyer, H. (2004). High rate of spontaneous clearance of acute hepatitis C virus genotype 3 infection. *J Med Virol* **73**, 387-391.

Lenschow, D. J., Walunas, T. L. & Bluestone, J. A. (1996). CD28/B7 system of T cell costimulation. *Annu Rev Immunol* **14**, 233-258.

Lenzi, M., Johnson, P. J., McFarlane, I. G., Ballardini, G., Smith, H. M., McFarlane, B. M., Bridger, C., Vergani, D., Bianchi, F. B. & Williams, R. (1991). Antibodies to hepatitis C virus in autoimmune liver disease: evidence for geographical heterogeneity. *Lancet* **338**, 277-280.

Lerat, H., Rumin, S., Habersetzer, F., Berby, F., Trabaud, M. A., Trepo, C. & Inchauspe, G. (1998). In vivo tropism of hepatitis C virus genomic sequences in hematopoietic cells: influence of viral load, viral genotype, and cell phenotype. *Blood* **91**, 3841-3849.

Levels, J. H., Abraham, P. R., van den Ende, A. & van Deventer, S. J. (2001). Distribution and kinetics of lipoprotein-bound endotoxin. *Infect Immun* **69**, 2821-2828.

Levitskaya, J., Coram, M., Levitsky, V., Imreh, S., Steigerwald-Mullen, P. M., Klein, G., Kurilla, M. G. & Masucci, M. G. (1995). Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. *Nature* **375**, 685-688.

Levy, S. & Shoham, T. (2005). The tetraspanin web modulates immune-signalling complexes. *Nat Rev Immunol* **5**, 136-148.

Levy, S., Todd, S. C. & Maecker, H. T. (1998). CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. *Annu Rev Immunol* **16**, 89-109.

Li, K., Foy, E., Ferreon, J. C., Nakamura, M., Ferreon, A. C., Ikeda, M., Ray, S. C., Gale, M., Jr. & Lemon, S. M. (2005). Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc Natl Acad Sci U S A* **102**, 2992-2997. Epub 2005 Feb 2914.

Liberman, E., Fong, Y. L., Selby, M. J., Choo, Q. L., Cousens, L., Houghton, M. & Yen, T. S. (1999). Activation of the grp78 and grp94 promoters by hepatitis C virus E2 envelope protein. *J Virol* **73**, 3718-3722.

Lin, C., Lindenbach, B. D., Pragai, B. M., McCourt, D. W. & Rice, C. M. (1994). Processing in the hepatitis C virus E2-NS2 region: identification of p7 and two distinct E2-specific products with different C termini. *J Virol* **68**, 5063-5073.

Lin, W., Choe, W. H., Hiasa, Y., Kamegaya, Y., Blackard, J. T., Schmidt, E. V. & Chung, R. T. (2005). Hepatitis C virus expression suppresses interferon signaling by degrading STAT1. *Gastroenterology* **128**, 1034-1041.

Littaua, R., Kurane, I. & Ennis, F. A. (1990). Human IgG Fc receptor II mediates antibody-dependent enhancement of dengue virus infection. *J Immunol* **144**, 3183-3186.

Liu, Q., Tackney, C., Bhat, R. A., Prince, A. M. & Zhang, P. (1997). Regulated processing of hepatitis C virus core protein is linked to subcellular localization. *J Virol* **71**, 657-662.

Liu, Z. X., Nishida, H., He, J. W., Lai, M. M., Feng, N. & Dennert, G. (2002). Hepatitis C virus genotype 1b core protein does not exert immunomodulatory effects on virus-induced cellular immunity. *J Virol* **76**, 990-997.

Ljunggren, H. G. & Karre, K. (1990). In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today* **11**, 237-244.

Lo, S. Y., Selby, M. J. & Ou, J. H. (1996). Interaction between hepatitis C virus core protein and E1 envelope protein. *J Virol* **70**, 5177-5182.

Lobigs, M., Blanden, R. V. & Mullbacher, A. (1996). Flavivirus-induced up-regulation of MHC class I antigens; implications for the induction of CD8+ T-cell-mediated autoimmunity. *Immunol Rev* **152**, 5-19.

Loh, J., Chu, D. T., O'Guin, A. K., Yokoyama, W. M. & Virgin, H. W. t. (2005). Natural killer cells utilize both perforin and gamma interferon to regulate murine cytomegalovirus infection in the spleen and liver. *J Virol* **79**, 661-667.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L. & Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110-113.

Long, E. O. (1999). Regulation of immune responses through inhibitory receptors. *Annu Rev Immunol* **17**, 875-904.

Longman, R. S., Talal, A. H., Jacobson, I. M., Albert, M. L. & Rice, C. M. (2004). Presence of functional dendritic cells in patients chronically infected with hepatitis C virus. *Blood* **103**, 1026-1029. Epub 2003 Oct 1022.

Lozach, P. Y., Amara, A., Bartosch, B., Virelizier, J. L., Arenzana-Seisdedos, F., Cosset, F. L. & Altmeyer, R. (2004). C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus pseudotype particles. *J Biol Chem* **279**, 32035-32045. Epub 32004 May 32027.

Lozach, P. Y., Lortat-Jacob, H., de Lacroix de Lavalette, A., Staropoli, I., Foung, S., Amara, A., Houles, C., Fieschi, F., Schwartz, O., Virelizier, J. L., Arenzana-Seisdedos, F. & Altmeyer, R. (2003). DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. *J Biol Chem* **278**, 20358-20366. Epub 22003 Feb 20327.

Lucas, M., Vargas-Cuero, A. L., Lauer, G. M., Barnes, E., Willberg, C. B., Semmo, N., Walker, B. D., Phillips, R. & Klenerman, P. (2004). Pervasive influence of hepatitis C virus on the phenotype of antiviral CD8+ T cells. *J Immunol* **172**, 1744-1753.

Lucas, M., Tsitoura, E., Montoya, M., Laliotou, B., Aslanoglou, E., Kouvatsis, V., Entwisle, C., Miller, J., Klenerman, P., Hadziyannis, A., Hadziyannis, S., Borrow, P. & Mavromara, P. (2003). Characterization of secreted and intracellular forms of a truncated hepatitis C virus E2 protein expressed by a recombinant herpes simplex virus. *J Gen Virol* **84**, 545-554.

Ludwig, I. S., Lekkerkerker, A. N., Depla, E., Bosman, F., Musters, R. J., Depraetere, S., van Kooyk, Y. & Geijtenbeek, T. B. (2004). Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation. *J Virol* **78**, 8322-8332.

Macdonald, A. & Harris, M. (2004). Hepatitis C virus NS5A: tales of a promiscuous protein. *J Gen Virol* **85**, 2485-2502.

MacDonald, K. P., Munster, D. J., Clark, G. J., Dzionek, A., Schmitz, J. & Hart, D. N. (2002). Characterization of human blood dendritic cell subsets. *Blood* **100**, 4512-4520. Epub 2002 Aug 4515.

Machida, K., Cheng, K. T., Pavio, N., Sung, V. M. & Lai, M. M. (2005). Hepatitis C virus E2-CD81 interaction induces hypermutation of the immunoglobulin gene in B cells. *J Virol* **79**, 8079-8089.

Mackenzie, J. M. & Westaway, E. G. (2001). Assembly and maturation of the flavivirus Kunjin virus appear to occur in the rough endoplasmic reticulum and along the secretory pathway, respectively. *J Virol* **75**, 10787-10799.

Maecker, H. T. & Levy, S. (1997). Normal lymphocyte development but delayed humoral immune response in CD81-null mice. *Journal of Experimental Medicine* **185**, 1505-1510.

Maecker, H. T., Todd, S. C. & Levy, S. (1997). The tetraspanin superfamily: molecular facilitators. *Faseb J* **11**, 428-442.

Maecker, H. T., Do, M. S. & Levy, S. (1998). CD81 on B cells promotes interleukin 4 secretion and antibody production during T helper type 2 immune responses. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 2458-2462.

Maecker, H. T., Todd, S. C., Kim, E. C. & Levy, S. (2000). Differential expression of murine CD81 highlighted by new anti-mouse CD81 monoclonal antibodies. *Hybridoma* **19**, 15-22.

Maeda, N., Watanabe, M., Okamoto, S., Kanai, T., Yamada, T., Hata, J., Hozumi, N., Katsume, A., Nuriya, H., Sandhu, J., Ishii, H., Kohara, M. & Hibi, T. (2004). Hepatitis C virus infection in human liver tissue engrafted in mice with an infectious molecular clone. *Liver Int* **24**, 259-267.

Majumder, M., Ghosh, A. K., Steele, R., Zhou, X. Y., Phillips, N. J., Ray, R. & Ray, R. B. (2002). Hepatitis C virus NS5A protein impairs TNF-mediated hepatic apoptosis, but not by an anti-FAS antibody, in transgenic mice. *Virology* **294**, 94-105.

Mannion, B. A., Berditchevski, F., Kraeft, S. K., Chen, L. B. & Hemler, M. E. (1996). Transmembrane-4 superfamily proteins CD81 (TAPA-1), CD82, CD63, and CD53 specifically associate with integrin alpha(4)beta(1) (CD49d/CD29). *Journal of Immunology* **157**, 2039-2047.

Marcellin, P. (1999). Hepatitis C: the clinical spectrum of the disease. *J Hepatol* **31**, 9-16.

Masciopinto, F., Freer, G., Burgio, V. L., Levy, S., Galli-Stampino, L., Bendinelli, M., Houghton, M., Abrignani, S. & Uematsu, Y. (2002). Expression of human CD81 in transgenic mice does not confer susceptibility to hepatitis C virus infection. *Virology* **304**, 187-196.

Masciopinto, F., Giovani, C., Campagnoli, S., Galli-Stampino, L., Colombatto, P., Brunetto, M., Yen, T. S., Houghton, M., Pileri, P. & Abrignani, S. (2004). Association of hepatitis C virus envelope proteins with exosomes. *Eur J Immunol* **34**, 2834.

Matsumoto, M., Funami, K., Tanabe, M., Oshiumi, H., Shingai, M., Seto, Y., Yamamoto, A. & Seya, T. (2003). Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J Immunol* **171**, 3154-3162.

Matsuura, Y., Suzuki, T., Suzuki, R., Sato, M., Aizaki, H., Saito, I. & Miyamura, T. (1994). Processing of E1 and E2 Glycoproteins of Hepatitis-C Virus Expressed in Mammalian and Insect Cells. *Virology* **205**, 141-150.

Matsuura, Y., Tani, H., Suzuki, K., Kimura-Someya, T., Suzuki, R., Aizaki, H., Ishii, K., Moriishi, K., Robison, C. S., Whitt, M. A. & Miyamura, T. (2001). Characterization of pseudotype VSV possessing HCV envelope proteins. *Virology* **286**, 263-275.

Mattapallil, J. J., Douek, D. C., Hill, B., Nishimura, Y., Martin, M. & Roederer, M. (2005). Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* **434**, 1093-1097.

McKeating, J. A., Zhang, L. Q., Logvinoff, C., Flint, M., Zhang, J., Yu, J., Butera, D., Ho, D. D., Dustin, L. B., Rice, C. M. & Balfe, P. (2004). Diverse hepatitis C virus glycoproteins mediate viral infection in a CD81-dependent manner. *J Virol* **78**, 8496-8505.

McKechnie, V. M., Mills, P. R. & McCruden, E. A. (2000). The NS5a gene of hepatitis C virus in patients treated with interferon-alpha. *J Med Virol* **60**, 367-378.

McLauchlan, J. (2000). Properties of the hepatitis C virus core protein: a structural protein that modulates cellular processes. *J Viral Hepat* **7**, 2-14.

McLauchlan, J., Lemberg, M. K., Hope, G. & Martoglio, B. (2002). Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *Embo J* **21**, 3980-3988.

McMahon, C. W., Zajac, A. J., Jamieson, A. M., Corral, L., Hammer, G. E., Ahmed, R. & Raulet, D. H. (2002). Viral and bacterial infections induce expression of multiple NK cell receptors in responding CD8(+) T cells. *J Immunol* **169**, 1444-1452.

Medzhitov, R. & Janeway, C. A., Jr. (1997). Innate immunity: impact on the adaptive immune response. *Curr Opin Immunol* **9**, 4-9.

Medzhitov, R., Preston-Hurlburt, P. & Janeway, C. A., Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388**, 394-397.

Megiovanni, A. M., Sanchez, F., Gluckman, J. C. & Rosenzwajg, M. (2004). Double-stranded RNA stimulation or CD40 ligation of monocyte-derived dendritic cells as models to study their activation and maturation process. *Eur Cytokine Netw* **15**, 126-134.

Mehal, W. Z., Azzaroli, F. & Crispe, I. N. (2001). Immunology of the healthy liver: old questions and new insights. *Gastroenterology* **120**, 250-260.

Meier, U., Owen, R. E., Taylor, E., Worth, A., Naoumov, N., Willberg, C., Tang, K., Newton, P., Pellegrino, P., Williams, I., Klenerman, P. & Borrow, P. (2005). Shared alternations in NK cell frequency, phenotype, and function in chronic human immunodficiency virus and hepatitis C virus infections. *Journal of Virology* **79**.

Memon, M. I. & Memon, M. A. (2002). Hepatitis C: an epidemiological review. *J Viral Hepat* **9**, 84-100.

Meola, A., Sbardellati, A., Bruni Ercole, B., Cerretani, M., Pezzanera, M., Ceccacci, A., Vitelli, A., Levy, S., Nicosia, A., Traboni, C., McKeating, J. & Scarselli, E. (2000). Binding of hepatitis C virus E2 glycoprotein to CD81 does not correlate with species permissiveness to infection. *J Virol* **74**, 5933-5938.

Mercer, D. F., Schiller, D. E., Elliott, J. F., Douglas, D. N., Hao, C., Rinfret, A., Addison, W. R., Fischer, K. P., Churchill, T. A., Lakey, J. R., Tyrrell, D. L. & Kneteman, N. M. (2001). Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* **7**, 927-933.

Metelitsa, L. S., Naidenko, O. V., Kant, A., Wu, H. W., Loza, M. J., Perussia, B., Kronenberg, M. & Seeger, R. C. (2001). Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. *J Immunol* **167**, 3114-3122.

Metes, D., Manciulea, M., Pretrusca, D., Rabinowich, H., Ernst, L. K., Popescu, I., Calugaru, A., Sulica, A., Chambers, W. H., Herberman, R. B. & Morel, P. A. (1999). Ligand binding specificities and signal transduction pathways of Fc gamma

receptor IIc isoforms: the CD32 isoforms expressed by human NK cells. *Eur J Immunol* **29**, 2842-2852.

Meunier, J. C., Engle, R. E., Faulk, K., Zhao, M., Bartosch, B., Alter, H., Emerson, S. U., Cosset, F. L., Purcell, R. H. & Bukh, J. (2005). Evidence for cross-genotype neutralization of hepatitis C virus pseudo-particles and enhancement of infectivity by apolipoprotein C1. *Proc Natl Acad Sci U S A* **102**, 4560-4565. Epub 2005 Mar 4514.

Meyer zum Bueschenfelde, C. O., Unternaehrer, J., Mellman, I. & Bottomly, K. (2004). Regulated recruitment of MHC class II and costimulatory molecules to lipid rafts in dendritic cells. *J Immunol* **173**, 6119-6124.

Michalak, J. P., Wychowski, C., Choukhi, A., Meunier, J. C., Ung, S., Rice, C. M. & Dubuisson, J. (1997). Characterization of truncated forms of hepatitis C virus glycoproteins. *J Gen Virol* **78**, 2299-2306.

Mihm, U., Herrmann, E., Sarrazin, U., von Wagner, M., Kronenberger, B., Zeuzem, S. & Sarrazin, C. (2004). Association of serum interleukin-8 with virologic response to antiviral therapy in patients with chronic hepatitis C. *J Hepatol* **40**, 845-852.

Misiani, R., Bellavita, P., Fenili, D., Borelli, G., Marchesi, D., Massazza, M., Vendramin, G., Comotti, B., Tanzi, E., Scudeller, G. & et al. (1992). Hepatitis C virus infection in patients with essential mixed cryoglobulinemia. *Ann Intern Med* **117**, 573-577.

Missale, G., Bertoni, R., Lamonaca, V., Valli, A., Massari, M., Mori, C., Rumi, M. G., Houghton, M., Fiaccadori, F. & Ferrari, C. (1996). Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. *J Clin Invest* **98**, 706-714.

Mittelbrunn, M., Yanez-Mo, M., Sancho, D., Ursa, A. & Sanchez-Madrid, F. (2002). Cutting edge: dynamic redistribution of tetraspanin CD81 at the central zone of the immune synapse in both T lymphocytes and APC. *J Immunol* **169**, 6691-6695.

Miyake, K. (2004). Endotoxin recognition molecules, Toll-like receptor 4-MD-2. *Semin Immunol* **16**, 11-16.

Miyazaki, T., Muller, U. & Campbell, K. S. (1997). Normal development but differentially altered proliferative responses of lymphocytes in mice lacking CD81. *Embo J* **16**, 4217-4225.

Mondelli, M. U., Cerino, A., Segagni, L., Meola, A., Cividini, A., Silini, E. & Nicosia, A. (2001). Hypervariable region 1 of hepatitis C virus: immunological decoy or biologically relevant domain? *Antiviral Res* **52**, 153-159.

Monteverde, A., Ballare, M. & Pileri, S. (1997). Hepatic lymphoid aggregates in chronic hepatitis C and mixed cryoglobulinemia. *Springer Semin Immunopathol* **19**, 99-110.

Montoya, M., Edwards, M. J., Reid, D. M. & Borrow, P. (2005). Rapid activation of spleen dendritic cell subsets following lymphocytic choriomeningitis virus infection of mice: analysis of the involvement of type 1 IFN. *J Immunol* **174**, 1851-1861.

Montoya, M., Schiavoni, G., Mattei, F., Gresser, I., Belardelli, F., Borrow, P. & Tough, D. F. (2002). Type I interferons produced by dendritic cells promote their phenotypic and functional activation. *Blood* **99**, 3263-3271.

Moradpour, D., Englert, C., Wakita, T. & Wands, J. R. (1996). Characterization of cell lines allowing tightly regulated expression of hepatitis C virus core protein. *Virology* **222**, 51-63.

Moradpour, D., Grabscheid, B., Kammer, A. R., Schmidtke, G., Groettrup, M., Blum, H. E. & Cerny, A. (2001). Expression of hepatitis C virus proteins does not interfere with major histocompatibility complex class I processing and presentation in vitro. *Hepatology* **33**, 1282-1287.

Morelli, A. E., Larregina, A. T., Shufesky, W. J., Sullivan, M. L., Stolz, D. B., Papworth, G. D., Zahorchak, A. F., Logar, A. J., Wang, Z., Watkins, S. C., Falo, L. D., Jr. & Thomson, A. W. (2004). Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. *Blood* **104**, 3257-3266. Epub 2004 Jul 3229.

Moretta, L. & Moretta, A. (2004). Unravelling natural killer cell function: triggering and inhibitory human NK receptors. *Embo J* **23**, 255-259. Epub 2003 Dec 2018.

Moser, J. M., Gibbs, J., Jensen, P. E. & Lukacher, A. E. (2002). CD94-NKG2A receptors regulate antiviral CD8+T cell responses. *Nature Immunology* **3**, 189-195.

Moser, M. & Murphy, K. M. (2000). Dendritic cell regulation of TH1-TH2 development. *Nat Immunol* **1**, 199-205.

Mottola, G., Cardinali, G., Ceccacci, A., Trozzi, C., Bartholomew, L., Torrisi, M. R., Pedrazzini, E., Bonatti, S. & Migliaccio, G. (2002). Hepatitis C virus nonstructural proteins are localized in a modified endoplasmic reticulum of cells expressing viral subgenomic replicons. *Virology* **293**, 31-43.

Muchmore, E., Popper, H., Peterson, D. A., Miller, M. F. & Lieberman, H. M. (1988). Non-A, non-B hepatitis-related hepatocellular carcinoma in a chimpanzee. *J Med Primatol* **17**, 235-246.

Muller, H. M., Pfaff, E., Goeser, T., Kallinowski, B., Solbach, C. & Theilmann, L. (1993). Peripheral blood leukocytes serve as a possible extrahepatic site for hepatitis C virus replication. *J Gen Virol* **74**, 669-676.

Murao, K., Terpstra, V., Green, S. R., Kondratenko, N., Steinberg, D. & Quehenberger, O. (1997). Characterization of CLA-1, a human homologue of rodent scavenger receptor BI, as a receptor for high density lipoprotein and apoptotic thymocytes. *J Biol Chem* **272**, 17551-17557.

Nakajima, H., Cocquerel, L., Kiyokawa, N., Fujimoto, J. & Levy, S. (2005). Kinetics of HCV envelope proteins' interaction with CD81 large extracellular loop. *Biochem Biophys Res Commun* **328**, 1091-1100.

Nakamura, I., Kajino, K., Bamba, H., Itoh, F., Takikita, M. & Ogasawara, K. (2004). Phenotypic stability of mature dendritic cells tuned by TLR or CD40 to control the efficiency of cytotoxic T cell priming. *Microbiol Immunol* **48**, 211-219.

Nakano, H., Yanagita, M. & Gunn, M. D. (2001). CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *J Exp Med* **194**, 1171-1178.

Nattermann, J., Nischalke, H. D., Feldmann, G., Ahlenstiel, G., Sauerbruch, T. & Spengler, U. (2004). Binding of HCV E2 to CD81 induces RANTES secretion and internalization of CC chemokine receptor 5. *J Viral Hepat* **11**, 519-526.

Nattermann, J., Hofmeister, V., Nischalke, H. D., Weiss, E., Houghton, M., Sauerbruch, T. & Spengler, U. (2003). Binding of HCV E2 to CD81 enhances surface expression of HLA-E. *Journal of Hepatology* **38**, 393.

Nattermann, J., Nischalke, H. D., Hofmeister, V., Ahlenstiel, G., Zimmermann, H., Leifeld, L., Weiss, E. H., Sauerbruch, T. & Spengler, U. (2005). The HLA-A2 restricted T cell epitope HCV core 35-44 stabilizes HLA-E expression and inhibits cytolysis mediated by natural killer cells. *Am J Pathol* **166**, 443-453.

Navas, M. C., Fuchs, A., Schvoerer, E., Bohbot, A., Aubertin, A. M. & Stoll-Keller, F. (2002). Dendritic cell susceptibility to hepatitis C virus genotype 1 infection. *J Med Virol* **67**, 152-161.

Neil, S., Martin, F., Ikeda, Y. & Collins, M. (2001). Postentry restriction to human immunodeficiency virus-based vector transduction in human monocytes. *J Virol* **75**, 5448-5456.

Nelson, D. R., Marousis, C. G., Davis, G. L., Rice, C. M., Wong, J., Houghton, M. & Lau, J. Y. N. (1997). The role of hepatitis C virus-specific cytotoxic T lymphocytes in chronic hepatitis C. *Journal of Immunology* **158**, 1473-1481.

Nguyen, K. B., Salazar-Mather, T. P., Dalod, M. Y., Van Deusen, J. B., Wei, X. Q., Liew, F. Y., Caligiuri, M. A., Durbin, J. E. & Biron, C. A. (2002). Coordinated and distinct roles for IFN-alpha beta, IL-12, and IL-15 regulation of NK cell responses to viral infection. *J Immunol* **169**, 4279-4287.

Ni, J., Hembrador, E., Di Bisceglie, A. M., Jacobson, I. M., Talal, A. H., Butera, D., Rice, C. M., Chambers, T. J. & Dustin, L. B. (2003). Accumulation of B lymphocytes with a naive, resting phenotype in a subset of hepatitis C patients. *J Immunol* **170**, 3429-3439.

Okazaki, I. M., Hiai, H., Kakazu, N., Yamada, S., Muramatsu, M., Kinoshita, K. & Honjo, T. (2003). Constitutive expression of AID leads to tumorigenesis. *J Exp Med* **197**, 1173-1181.

Okuda, M., Hino, K., Korenaga, M., Yamaguchi, Y., Katoh, Y. & Okita, K. (1999). Differences in hypervariable region 1 quasispecies of hepatitis C virus in human serum, peripheral blood mononuclear cells, and liver. *Hepatology* **29**, 217-222.

Op De Beeck, A., Montserret, R., Duvet, S., Cocquerel, L., Cacan, R., Barberot, B., Le Maire, M., Penin, F. & Dubuisson, J. (2000). The transmembrane domains of hepatitis C virus envelope glycoproteins E1 and E2 play a major role in heterodimerization. *J Biol Chem* **275**, 31428-31437.

Op De Beeck, A., Voisset, C., Bartosch, B., Ciczora, Y., Cocquerel, L., Keck, Z., Foung, S., Cosset, F. L. & Dubuisson, J. (2004). Characterization of functional hepatitis C virus envelope glycoproteins. *J Virol* **78**, 2994-3002.

Oren, R., Takahashi, S., Doss, C., Levy, R. & Levy, S. (1990). TAPA-1, the target of an antiproliferative antibody, defines a new family of transmembrane proteins. *Mol Cell Biol* **10**, 4007-4015.

Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T. & Seya, T. (2003). TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat Immunol* **4**, 161-167. Epub 2003 Jan 2021.

O'Sullivan, B. & Thomas, R. (2003). CD40 and dendritic cell function. *Crit Rev Immunol* **23**, 83-107.

Otsuka, M., Kato, N., Moriyama, M., Taniguchi, H., Wang, Y., Dharel, N., Kawabe, T. & Omata, M. (2005). Interaction between the HCV NS3 protein and the host TBK1 protein leads to inhibition of cellular antiviral responses. *Hepatology* **41**, 1004-1012.

Owsianka, A., Clayton, R. F., Loomis-Price, L. D., McKeating, J. A. & Patel, A. H. (2001). Functional analysis of hepatitis C virus E2 glycoproteins and virus-like particles reveals structural dissimilarities between different forms of E2. *J Gen Virol* **82**, 1877-1883.

Pang, P. S., Jankowsky, E., Planet, P. J. & Pyle, A. M. (2002). The hepatitis C viral NS3 protein is a processive DNA helicase with cofactor enhanced RNA unwinding. *Embo J* **21**, 1168-1176.

Parham, P. (2004). Immunology. NK cells lose their inhibition. *Science* **305**, 786-787.

Park, S. H., Chiu, Y. H., Jayawardena, J., Roark, J., Kavita, U. & Bendelac, A. (1998). Innate and adaptive functions of the CD1 pathway of antigen presentation. *Semin Immunol* **10**, 391-398.

Patel, A. H., Wood, J., Penin, F., Dubuisson, J. & McKeating, J. A. (2000). Construction and characterization of chimeric hepatitis C virus E2 glycoproteins: analysis of regions critical for glycoprotein aggregation and CD81 binding. *J Gen Virol* **81**, 2873-2883.

Patel, J., Patel, A. H. & McLauchlan, J. (2001). The transmembrane domain of the hepatitis C virus E2 glycoprotein is required for correct folding of the E1 glycoprotein and native complex formation. *Virology* **279**, 58-68.

Patel, M., Yanagishita, M., Roderiquez, G., Bou-Habib, D. C., Oravecz, T., Hascall, V. C. & Norcross, M. A. (1993). Cell-surface heparan sulfate proteoglycan mediates HIV-1 infection of T-cell lines. *AIDS Res Hum Retroviruses* **9**, 167-174.

Pavio, N. & Lai, M. M. (2003). The hepatitis C virus persistence: how to evade the immune system? *J Biosci* **28**, 287-304.

Pavio, N., Romano, P. R., Graczyk, T. M., Feinstone, S. M. & Taylor, D. R. (2003). Protein synthesis and endoplasmic reticulum stress can be modulated by the hepatitis C virus envelope protein E2 through the eukaryotic initiation factor 2alpha kinase PERK. *J Virol* **77**, 3578-3585.

Pawlotsky, J. M., Ben Yahia, M., Andre, C., Voisin, M. C., Intrator, L., Roudot-Thoraval, F., Deforges, L., Duvoux, C., Zafrani, E. S., Duval, J. & et al. (1994). Immunological disorders in C virus chronic active hepatitis: a prospective case-control study. *Hepatology* **19**, 841-848.

Pearlman, B. L. (2004). Hepatitis C treatment update. *Am J Med* **117**, 344-352.

Pearson, A. M. (1996). Scavenger receptors in innate immunity. *Curr Opin Immunol* **8**, 20-28.

Peiris, J. S. & Porterfield, J. S. (1979). Antibody-mediated enhancement of Flavivirus replication in macrophage-like cell lines. *Nature* **282**, 509-511.

Pellicano, R., Mladenova, I., Dimitrova, S. M., Bruno, C. M., Sciacca, C. & Rizzetto, M. (2004). The epidemiology of hepatitis C virus infection. An update for clinicians. *Minerva Gastroenterol Dietol* **50**, 1-7.

Pende, D., Cantoni, C., Rivera, P., Vitale, M., Castriconi, R., Marcenaro, S., Nanni, M., Biassoni, R., Bottino, C., Moretta, A. & Moretta, L. (2001). Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of nonepithelial origin. *Eur J Immunol* **31**, 1076-1086.

Penin, F., Combet, C., Germanidis, G., Frainais, P. O., Deleage, G. & Pawlotsky, J. M. (2001). Conservation of the conformation and positive charges of hepatitis C virus E2 envelope glycoprotein hypervariable region 1 points to a role in cell attachment. *J Virol* **75**, 5703-5710.

Petracca, R., Falugi, F., Galli, G., Norais, N., Rosa, D., Campagnoli, S., Burgio, V., Di Stasio, E., Giardina, B., Houghton, M., Abrignani, S. & Grandi, G. (2000). Structure-function analysis of hepatitis C virus envelope-CD81 binding. *Journal of Virology* **74**, 4824-4830.

Pfeiffer, A., Bottcher, A., Orso, E., Kapinsky, M., Nagy, P., Bodnar, A., Spreitzer, I., Liebisch, G., Drobnik, W., Gempel, K., Horn, M., Holmer, S., Hartung, T., Multhoff, G., Schutz, G., Schindler, H., Ulmer, A. J., Heine, H., Stelter, F., Schutt, C., Rothe, G., Szollosi, J., Damjanovich, S. & Schmitz, G. (2001). Lipopolysaccharide and ceramide docking to CD14 provokes ligand-specific receptor clustering in rafts. *Eur J Immunol* **31**, 3153-3164.

Pflugheber, J., Fredericksen, B., Sumpter, R., Jr., Wang, C., Ware, F., Sodora, D. L. & Gale, M., Jr. (2002). Regulation of PKR and IRF-1 during hepatitis C virus RNA replication. *Proc Natl Acad Sci USA* **99**, 4650-4655. Epub 2002 Mar 4619.

Piccioli, D., Sbrana, S., Melandri, E. & Valiante, N. M. (2002). Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. *J Exp Med* **195**, 335-341.

Piccioli, D., Tavarini, S., Nuti, S., Colombatto, P., Brunetto, M., Bonino, F., Ciccorossi, P., Zorat, F., Pozzato, G., Comar, C., Abrignani, S. & Wack, A. (2005). Comparable functions of plasmacytoid and monocyte-derived dendritic cells in chronic hepatitis C patients and healthy donors. *J Hepatol* **42**, 61-67.

Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., Weiner, A. J., Houghton, M., Rosa, D., Grandi, G. & Abrignani, S. (1998). Binding of hepatitis C virus to CD81. *Science* **282**, 938-941.

Planz, O., Seiler, P., Hengartner, H. & Zinkernagel, R. M. (1996). Specific cytotoxic T cells eliminate B cells producing virus-neutralizing antibodies [corrected]. *Nature* **382**, 726-729.

Pohlmann, S., Zhang, J., Baribaud, F., Chen, Z. W., Leslie, G., Lin, G., Granelli-Piperno, A., Dom, R. W., Rice, C. M. & McSKeating, J. A. (2003). Hepatitis C virus glycoproteins interact with DC-SIGN and DC- SIGNR. *Journal of Virology* **77**, 4070-4080.

Polyak, S. J., Khabar, K. S., Rezeiq, M. & Gretch, D. R. (2001a). Elevated levels of interleukin-8 in serum are associated with hepatitis C virus infection and resistance to interferon therapy. *J Virol* **75**, 6209-6211.

Polyak, S. J., Paschal, D. M., McArdle, S., Gale, M. J., Jr., Moradpour, D. & Gretch, D. R. (1999). Characterization of the effects of hepatitis C virus nonstructural 5A protein expression in human cell lines and on interferon-sensitive virus replication. *Hepatology* **29**, 1262-1271.

Polyak, S. J., Khabar, K. S., Paschal, D. M., Ezelle, H. J., Duverlie, G., Barber, G. N., Levy, D. E., Mukaida, N. & Gretch, D. R. (2001b). Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response. *J Virol* **75**, 6095-6106.

Prince, A. M., Brotman, B., Huima, T., Pascual, D., Jaffery, M. & Inchauspe, G. (1992). Immunity in hepatitis C infection. *J Infect Dis* **165**, 438-443.

Radkowski, M. & Laskus, T. (2005). Persistence of hepatitis C virus after successful treatment of chronic hepatitis C: is hepatitis C infection for life? *Liver Transpl* **11**, 114-116.

Radkowski, M., Wilkinson, J., Nowicki, M., Adair, D., Vargas, H., Ingui, C., Rakela, J. & Laskus, T. (2002). Search for hepatitis C virus negative-strand RNA sequences and analysis of viral sequences in the central nervous system: evidence of replication. *J Virol* **76**, 600-608.

Raftery, M. J., Wieland, D., Gronewald, S., Kraus, A. A., Giese, T. & Schonrich, G. (2004). Shaping phenotype, function, and survival of dendritic cells by cytomegalovirus-encoded IL-10. *J Immunol* **173**, 3383-3391.

Raposo, G., Nijman, H. W., Stoorvogel, W., Liejendekker, R., Harding, C. V., Melief, C. J. & Geuze, H. J. (1996). B lymphocytes secrete antigen-presenting vesicles. *J Exp Med* **183**, 1161-1172.

Ray, S. C., Wang, Y. M., Laeyendecker, O., Ticehurst, J. R., Villano, S. A. & Thomas, D. L. (1999). Acute hepatitis C virus structural gene sequences as predictors of persistent viremia: Hypervariable region 1 as a decoy. *Journal of Virology* **73**, 2938-2946.

Ray, S. C., Mao, Q., Lanford, R. E., Bassett, S., Laeyendecker, O., Wang, Y. M. & Thomas, D. L. (2000). Hypervariable region 1 sequence stability during hepatitis C virus replication in chimpanzees. *J Virol* **74**, 3058-3066.

Recher, M., Lang, K. S., Hunziker, L., Freigang, S., Eschli, B., Harris, N. L., Navarini, A., Senn, B. M., Fink, K., Lotscher, M., Hangartner, L., Zellweger, R., Hersberger, M., Theocharides, A., Hengartner, H. & Zinkernagel, R. M. (2004). Deliberate removal of T cell help improves virus-neutralizing antibody production. *Nat Immunol* **5**, 934-942. Epub 2004 Aug 2008.

Reed, K. E. & Rice, C. M. (2000). Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. *Curr Top Microbiol Immunol* **242**, 55-84.

Rehermann, B., Chang, K. M., McHutchinson, J., Kokka, R., Houghton, M., Rice, C. M. & Chisari, F. V. (1996). Differential cytotoxic T-lymphocyte responsiveness to the hepatitis B and C viruses in chronically infected patients. *J Virol* **70**, 7092-7102.

Reis e Sousa, C. (2001). Dendritic cells as sensors of infection. *Immunity* **14**, 495-498.

Reis e Sousa, C. (2004). Toll-like receptors and dendritic cells: for whom the bug tolls. *Semin Immunol* **16**, 27-34.

Rice, C. M. (1996). Flaviviridae: the viruses and their replication. In *Fields virology*, pp. 931-960. Edited by F. B. N. e. al. Philadelphia: Raven.

Rice, C. M. (1999). Is CD81 the key to hepatitis C virus entry? *Hepatology* **29**, 990-992.

Robertson, B., Myers, G., Howard, C., Brettin, T., Bukh, J., Gaschen, B., Gojobori, T., Maertens, G., Mizokami, M., Nainan, O., Netesov, S., Nishioka, K., Shin i, T., Simmonds, P., Smith, D., Stuyver, L. & Weiner, A. (1998). Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. International Committee on Virus Taxonomy. *Arch Virol* **143**, 2493-2503.

Roccasecca, R., Ansuini, H., Vitelli, A., Meola, A., Scarselli, E., Acali, S., Pezzanera, M., Ercole, B. B., McKeating, J., Yagnik, A., Lahm, A., Tramontano, A., Cortese, R. & Nicosia, A. (2003). Binding of the hepatitis C virus E2 glycoprotein to CD81 is strain specific and is modulated by a complex interplay between hypervariable regions 1 and 2. *J Virol* **77**, 1856-1867.

Roccasecca, R., Folgori, A., Ercole, B. B., Puntoriero, G., Lahm, A., Zucchelli, S., Tafi, R., Pezzanera, M., Galfre, G., Tramontano, A., Mondelli, M. U., Pessi, A., Nicosia, A., Cortese, R. & Meola, A. (2001). Induction of cross-reactive humoral immune response by immunization with mimotopes of the hypervariable region 1 of the hepatitis C virus. *Int Rev Immunol* **20**, 289-300.

Roe, T., Reynolds, T. C., Yu, G. & Brown, P. O. (1993). Integration of murine leukemia virus DNA depends on mitosis. *Embo J* **12**, 2099-2108.

Rollier, C., Drexhage, J. A., Verstrepen, B. E., Verschoor, E. J., Bontrop, R. E., Koopman, G. & Heeney, J. L. (2003). Chronic hepatitis C virus infection established and maintained in chimpanzees independent of dendritic cell impairment. *Hepatology* **38**, 851-858.

Rosa, D., Campagnoli, S., Moretto, C., Guenzi, E., Cousens, L., Chin, M., Dong, C., Weiner, A. J., Lau, J. Y., Choo, Q. L., Chien, D., Pileri, P., Houghton, M. & Abrignani, S. (1996). A quantitative test to estimate neutralizing antibodies to the hepatitis C virus: cytofluorimetric assessment of envelope glycoprotein 2 binding to target cells. *Proc Natl Acad Sci USA* **93**, 1759-1763.

Rosa, D. S., G. Pozzato, G. Abrignani, S. (2001).HCV activates B cells via CD81 engagement: a molecular mechanism for B cell autoreactivity and cryoglobulinemia in HCV infection. In *8th International Symposium on Hepatitis C Virus & Related Viruses*, pp. 34. Paris.

Rubbia-Brandt, L., Leandro, G., Spahr, L., Giostra, E., Quadri, R., Male, P. J. & Negro, F. (2001). Liver steatosis in chronic hepatitis C: a morphological sign suggesting infection with HCV genotype 3. *Histopathology* **39**, 119-124.

Rubinstein, E., Le Naour, F., Lagaudriere-Gesbert, C., Billard, M., Conjeaud, H. & Boucheix, C. (1996). CD9, CD63, CD81, and CD82 are components of a surface tetraspan network connected to HLA-DR and VLA integrins. *Eur J Immunol* **26**, 2657-2665.

Ruegg, C. L., Monell, C. R. & Strand, M. (1989). Identification, using synthetic peptides, of the minimum amino acid sequence from the retroviral transmembrane

protein p15E required for inhibition of lymphoproliferation and its similarity to gp21 of human T-lymphotropic virus types I and II. *J Virol* **63**, 3250-3256.

Saiz, J. C., Lopez-Labrador, F. X., Ampurdanes, S., Dopazo, J., Forns, X., Sanchez-Tapias, J. M. & Rodes, J. (1998). The prognostic relevance of the nonstructural 5A gene interferon sensitivity determining region is different in infections with genotype 1b and 3a isolates of hepatitis C virus. *J Infect Dis* **177**, 839-847.

Sakai, A., Claire, M. S., Faulk, K., Govindarajan, S., Emerson, S. U., Purcell, R. H. & Bukh, J. (2003). The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences. *Proc Natl Acad Sci USA* **100**, 11646-11651. Epub 12003 Sep 11622.

Salazar-Mather, T. P., Orange, J. S. & Biron, C. A. (1998). Early murine cytomegalovirus (MCMV) infection induces liver natural killer (NK) cell inflammation and protection through macrophage inflammatory protein 1alpha (MIP-1alpha)-dependent pathways. *J Exp Med* **187**, 1-14.

Samuel, C. E. (2001). Antiviral actions of interferons. *Clin Microbiol Rev* **14**, 778-809, table of contents.

Sandrin, V., Boson, B., Salmon, P., Gay, W., Negre, D., Le Grand, R., Trono, D. & Cosset, F. L. (2002). Lentiviral vectors pseudotyped with a modified RD114 envelope glycoprotein show increased stability in sera and augmented transduction of primary lymphocytes and CD34+ cells derived from human and nonhuman primates. *Blood* **100**, 823-832.

Sansonno, D., De Vita, S., Iacobelli, A. R., Cornacchiulo, V., Boiocchi, M. & Dammacco, F. (1998). Clonal analysis of intrahepatic B cells from HCV-infected patients with and without mixed cryoglobulinemia. *J Immunol* **160**, 3594-3601.

Santolini, E., Migliaccio, G. & La Monica, N. (1994). Biosynthesis and biochemical properties of the hepatitis C virus core protein. *J Virol* **68**, 3631-3641.

Santoro, F., Vassena, L. & Lusso, P. (2004). Chemokine receptors as new molecular targets for antiviral therapy. *New Microbiol* **27**, 17-29.

Sarobe, P., Lasarte, J. J., Casares, N., de Cerio, A. L. D., Baixeras, E., Labarga, P., Garcia, N., Borras-Cuesta, F. & Prieto, J. (2002). Abnormal priming of CD4(+) T cells by dendritic cells expressing hepatitis C virus core and E1 proteins. *Journal of Virology* **76**, 5062-5070.

Sato, K., Okamoto, H., Aihara, S., Hoshi, Y., Tanaka, T. & Mishiro, S. (1993). Demonstration of sugar moiety on the surface of hepatitis C virions recovered from the circulation of infected humans. *Virology* **196**, 354-357.

Scarselli, E., Ansuini, H., Cerino, R., Roccasecca, R. M., Acali, S., Filocamo, G., Traboni, C., Nicosia, A., Cortese, R. & Vitelli, A. (2002). The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *Embo J* **21**, 5017-5025.

Schick, M. R. & Levy, S. (1993). The TAPA-1 molecule is associated on the surface of B cells with HLA-DR molecules. *J Immunol* **151**, 4090-4097.

Schoenberger, S. P., Toes, R. E., van der Voort, E. I., Offringa, R. & Melief, C. J. (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* **393**, 480-483.

Schust, D. J., Tortorella, D., Seebach, J., Phan, C. & Ploegh, H. L. (1998). Trophoblast class I major histocompatibility complex (MHC) products are resistant to rapid degradation imposed by the human cytomegalovirus (HCMV) gene products US2 and US11. *J Exp Med* **188**, 497-503.

Schuttler, C. G., Thomas, C., Discher, T., Friese, G., Lohmeyer, J., Schuster, R., Schaefer, S. & Gerlich, W. H. (2004). Variable ratio of hepatitis C virus RNA to viral core antigen in patient sera. *J Clin Microbiol* **42**, 1977-1981.

Schuurhuis, D. H., Laban, S., Toes, R. E., Ricciardi-Castagnoli, P., Kleijmeer, M. J., van der Voort, E. I., Rea, D., Offringa, R., Geuze, H. J., Melief, C. J. & Ossendorp, F. (2000). Immature dendritic cells acquire CD8(+) cytotoxic T lymphocyte priming capacity upon activation by T helper cell-independent or -dependent stimuli. *J Exp Med* **192**, 145-150.

Secrist, H., Levy, S., DeKruyff, R. H. & Umetsu, D. T. (1996). Ligation of TAPA-1 (CD81) or major histocompatibility complex class II in co-cultures of human B and T lymphocytes enhances interleukin-4 synthesis by antigen-specific CD4+ T cells. *Eur J Immunol* **26**, 1435-1442.

Seifert, U., Liermann, H., Racanelli, V., Halenius, A., Wiese, M., Wedemeyer, H., Ruppert, T., Rispeter, K., Henklein, P., Sijts, A., Hengel, H., Kloetzel, P. M. & Rehermann, B. (2004). Hepatitis C virus mutation affects proteasomal epitope processing. *J Clin Invest* **114**, 250-259.

Shakil, A. O., Conry-Cantilena, C., Alter, H. J., Hayashi, P., Kleiner, D. E., Tedeschi, V., Krawczynski, K., Conjeevaram, H. S., Sallie, R. & Di Bisceglie, A. M. (1995). Volunteer blood donors with antibody to hepatitis C virus: clinical, biochemical, virologic, and histologic features. The Hepatitis C Study Group. *Ann Intern Med* **123**, 330-337.

Shellam, G. R., Allan, J. E., Papadimitriou, J. M. & Bancroft, G. J. (1981). Increased susceptibility to cytomegalovirus infection in beige mutant mice. *Proc Natl Acad Sci USA* **78**, 5104-5108.

Shen, Z., Reznikoff, G., Dranoff, G. & Rock, K. L. (1997). Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. *J Immunol* **158**, 2723-2730.

Shi, S. T., Lee, K. J., Aizaki, H., Hwang, S. B. & Lai, M. M. (2003). Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. *J Virol* **77**, 4160-4168.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K. & Kimoto, M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med* **189**, 1777-1782.

Shimizu, Y. K., Hijikata, M., Iwamoto, A., Alter, H. J., Purcell, R. H. & Yoshikura, H. (1994). Neutralizing antibodies against hepatitis C virus and the emergence of neutralization escape mutant viruses. *J Virol* **68**, 1494-1500.

Shimizu, Y. K., Igarashi, H., Kiyohara, T., Cabezon, T., Farci, P., Purcell, R. H. & Yoshikura, H. (1996). A hyperimmune serum against a synthetic peptide corresponding to the hypervariable region 1 of hepatitis C virus can prevent viral infection in cell cultures. *Virology* **223**, 409-412.

Shoham, T., Rajapaksa, R., Boucheix, C., Rubinstein, E., Poe, J. C., Tedder, T. F. & Levy, S. (2003). The tetraspanin CD81 regulates the expression of CD19 during B cell development in a postendoplasmic reticulum compartment. *J Immunol* **171**, 4062-4072.

Shoukry, N. H., Grakoui, A., Houghton, M., Chien, D. Y., Ghrayeb, J., Reimann, K. A. & Walker, C. M. (2003). Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection. *J Exp Med* **197**, 1645-1655.

Shreedhar, V., Moodycliffe, A. M., Ullrich, S. E., Bucana, C., Kripke, M. L. & Flores-Romo, L. (1999). Dendritic cells require T cells for functional maturation in vivo. *Immunity* **11**, 625-636.

Siegal, F. P., Kadowaki, N., Shodell, M., Fitzgerald-Bocarsly, P. A., Shah, K., Ho, S., Antonenko, S. & Liu, Y. J. (1999). The nature of the principal type 1 interferon-producing cells in human blood. *Science* **284**, 1835-1837.

Silver, D. L. & Tall, A. R. (2001). The cellular biology of scavenger receptor class B type I. *Curr Opin Lipidol* **12**, 497-504.

Silvie, O., Rubinstein, E., Franetich, J. F., Prenant, M., Belnoue, E., Renia, L., Hannoun, L., Eling, W., Levy, S., Boucheix, C. & Mazier, D. (2003). Hepatocyte CD81 is required for Plasmodium falciparum and Plasmodium yoelii sporozoite infectivity. *Nat Med* **9**, 93-96.

Simmonds, P., Holmes, E. C., Cha, T. A., Chan, S. W., McOmish, F., Irvine, B., Beall, E., Yap, P. L., Kolberg, J. & Urdea, M. S. (1993). Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. *J Gen Virol* **74**, 2391-2399.

Sivori, S., Pende, D., Bottino, C., Marcenaro, E., Pessino, A., Biassoni, R., Moretta, L. & Moretta, A. (1999). NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. *Eur J Immunol* **29**, 1656-1666.

Soilleux, E. J., Morris, L. S., Lee, B., Pohlmann, S., Trowsdale, J., Doms, R. W. & Coleman, N. (2001). Placental expression of DC-SIGN may mediate intrauterine vertical transmission of HIV. *J Pathol* **195**, 586-592.

Soilleux, E. J., Morris, L. S., Leslie, G., Chehimi, J., Luo, Q., Levroney, E., Trowsdale, J., Montaner, L. J., Doms, R. W., Weissman, D., Coleman, N. & Lee, B. (2002). Constitutive and induced expression of DC-SIGN on dendritic cell and macrophage subpopulations in situ and in vitro. *J Leukoc Biol* **71**, 445-457.

Soldaini, E., Wack, A., D'Oro, U., Nuti, S., Ulivieri, C., Baldari, C. T. & Abrignani, S. (2003). T cell costimulation by the hepatitis C virus envelope protein E2 binding to CD81 is mediated by Lck. *European Journal of Immunology* **33**, 455-464.

Sol-Foulon, N., Moris, A., Nobile, C., Boccaccio, C., Engering, A., Abastado, J. P., Heard, J. M., van Kooyk, Y. & Schwartz, O. (2002). HIV-1 Nef-induced upregulation of DC-SIGN in dendritic cells promotes lymphocyte clustering and viral spread. *Immunity* **16**, 145-155.

Song, E. S., Young, K. & Sears, D. W. (1990). Rat and human natural killers exhibit contrasting immunoglobulin G subclass specificities in antibody-dependent cellular cytotoxicity reflecting differences in their Fc receptors (Fc gamma R). *J Leukoc Biol* **48**, 524-530.

Spear, P. G. (2004). Herpes simplex virus: receptors and ligands for cell entry. *Cell Microbiol* **6**, 401-410.

Squadrito, G., Leone, F., Sartori, M., Nalpas, B., Berthelot, P., Raimondo, G., Pol, S. & Brechot, C. (1997). Mutations in the nonstructural 5A region of hepatitis C virus and response of chronic hepatitis C to interferon alfa. *Gastroenterology* **113**, 567-572.

Stehman-Breen, C., Willson, R., Alpers, C. E., Gretch, D. & Johnson, R. J. (1995). Hepatitis C virus-associated glomerulonephritis. *Curr Opin Nephrol Hypertens* **4**, 287-294.

Steinmann, D., Barth, H., Gissler, B., Schurmann, P., Adah, M. I., Gerlach, J. T., Pape, G. R., Depla, E., Jacobs, D., Maertens, G., Patel, A. H., Inchauspe, G., Liang, T. J., Blum, H. E. & Baumert, T. F. (2004). Inhibition of hepatitis C virus-like particle binding to target cells by antiviral antibodies in acute and chronic hepatitis C. *J Virol* **78**, 9030-9040.

Stipp, C. S., Kolesnikova, T. V. & Hemler, M. E. (2001). EWI-2 is a major CD9 and CD81 partner and member of a novel Ig protein subfamily. *J Biol Chem* **276**, 40545-40554. Epub 42001 Aug 40514.

Stipp, C. S., Kolesnikova, T. V. & Hemler, M. E. (2003a). Functional domains in tetraspanin proteins. *Trends Biochem Sci* **28**, 106-112.

Stipp, C. S., Kolesnikova, T. V. & Hemler, M. E. (2003b). EWI-2 regulates alpha3beta1 integrin-dependent cell functions on laminin-5. *J Cell Biol* **163**, 1167-1177.

Su, A. I., Pezacki, J. P., Wodicka, L., Brideau, A. D., Supekova, L., Thimme, R., Wieland, S., Bukh, J., Purcell, R. H., Schultz, P. G. & Chisari, F. V. (2002). Genomic analysis of the host response to hepatitis C virus infection. *Proc Natl Acad Sci USA* **99**, 15669-15674. Epub 12002 Nov 15619.

Sugimoto, K., Ikeda, F., Stadanlick, J., Nunes, F. A., Alter, H. J. & Chang, K. M. (2003). Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. *Hepatology* **38**, 1437-1448.

Sulica, A., Gherman, M., Galatiuc, C., Manciulea, M. & Herberman, R. B. (1982). Inhibition of human natural killer cell activity by cytophilic immunoglobulin G. *J Immunol* **128**, 1031-1036.

Sulica, A., Metes, D., Gherman, M., Whiteside, T. L. & Herberman, R. B. (1996). Divergent effects of Fc gamma RIIIA ligands on the functional activities of human natural killer cells in vitro. *Eur J Immunol* **26**, 1199-1203.

Sulica, A., Galatiuc, C., Manciulea, M., Bancu, A. C., DeLeo, A., Whiteside, T. L. & Herberman, R. B. (1993). Regulation of human natural cytotoxicity by IgG. IV. Association between binding of monomeric IgG to the Fc receptors on large granular lymphocytes and inhibition of natural killer (NK) cell activity. *Cell Immunol* **147**, 397-410.

Sun, J. C. & Bevan, M. J. (2003). Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science* **300**, 339-342.

Sung, V. M. & Lai, M. M. (2002). Murine retroviral pseudotype virus containing hepatitis B virus large and small surface antigens confers specific tropism for primary human hepatocytes: a potential liver-specific targeting system. *J Virol* **76**, 912-917.

Sutherland, C. L., Chalupny, N. J., Schooley, K., VandenBos, T., Kubin, M. & Cosman, D. (2002). UL16-binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells. *J Immunol* **168**, 671-679.

Szollosi, J., Horejsi, V., Bene, L., Angelisova, P. & Damjanovich, S. (1996). Supramolecular complexes of MHC class I, MHC class II, CD20, and tetraspan molecules (CD53, CD81, and CD82) at the surface of a B cell line JY. *J Immunol* **157**, 2939-2946.

Szymanski, I. O., Pullman, J. M. & Underwood, J. M. (1994). Electron microscopic and immunochemical studies in a patient with hepatitis C virus infection and mixed cryoglobulinemia type II. *Am J Clin Pathol* **102**, 278-283.

Tai, X. G., Toyooka, K., Yashiro, Y., Abe, R., Park, C. S., Hamaoka, T., Kobayashi, M., Neben, S. & Fujiwara, H. (1997). CD9-mediated costimulation of TCR-triggered naive T cells leads to activation followed by apoptosis. *J Immunol* **159**, 3799-3807.

Takaki, A., Wiese, M., Maertens, G., Depla, E., Seifert, U., Liebetrau, A., Miller, J. L., Manns, M. P. & Rehermann, B. (2000). Cellular immune responses persist and

humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. *Nat Med* **6**, 578-582.

Takeda, K. & Akira, S. (2004). TLR signaling pathways. *Semin Immunol* **16**, 3-9.

Takikawa, S., Ishii, K., Aizaki, H., Suzuki, T., Asakura, H., Matsuura, Y. & Miyamura, T. (2000). Cell fusion activity of hepatitis C virus envelope proteins. *Journal of Virology* **74**, 5066-5074.

Tardif, K. D. & Siddiqui, A. (2003). Cell surface expression of major histocompatibility complex class I molecules is reduced in hepatitis C virus subgenomic replicon-expressing cells. *J Virol* **77**, 11644-11650.

Tardif, K. D., Mori, K. & Siddiqui, A. (2002). Hepatitis C virus subgenomic replicons induce endoplasmic reticulum stress activating an intracellular signaling pathway. *J Virol* **76**, 7453-7459.

Tardif, K. D., Waris, G. & Siddiqui, A. (2005). Hepatitis C virus, ER stress, and oxidative stress. *Trends Microbiol* **13**, 159-163.

Tarrant, J. M., Robb, L., van Spriel, A. B. & Wright, M. D. (2003). Tetraspanins: molecular organisers of the leukocyte surface. *Trends Immunol* **24**, 610-617.

Tassaneetrithep, B., Burgess, T. H., Granelli-Piperno, A., Trumpfheller, C., Finke, J., Sun, W., Eller, M. A., Pattanapanyasat, K., Sarasombath, S., Birx, D. L., Steinman, R. M., Schlesinger, S. & Marovich, M. A. (2003). DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J Exp Med* **197**, 823-829.

Taylor, D. R., Shi, S. T. & Lai, M. M. (2000). Hepatitis C virus and interferon resistance. *Microbes Infect* **2**, 1743-1756.

Taylor, D. R., Shi, S. T., Romano, P. R., Barber, G. N. & Lai, M. M. C. (1999). Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. *Science* **285**, 107-110.

Terheyden, P., Straten, P., Brocker, E. B., Kampgen, E. & Becker, J. C. (2000). CD40-ligated dendritic cells effectively expand melanoma-specific CD8+ CTLs and CD4+ IFN-gamma-producing T cells from tumor-infiltrating lymphocytes. *J Immunol* **164**, 6633-6639.

Thimme, R., Oldach, D., Chang, K. M., Steiger, C., Ray, S. C. & Chisari, F. V. (2001). Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med* **194**, 1395-1406.

Thimme, R., Bukh, J., Spangenberg, H. C., Wieland, S., Pemberton, J., Steiger, C., Govindarajan, S., Purcell, R. H. & Chisari, F. V. (2002). Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proc Natl Acad Sci USA* **99**, 15661-15668. Epub 12002 Nov 15619.

Thomson, M., Nascimbeni, M., Havert, M. B., Major, M., Gonzales, S., Alter, H., Feinstone, S. M., Murthy, K. K., Rehermann, B. & Liang, T. J. (2003). The clearance of hepatitis C virus infection in chimpanzees may not necessarily correlate with the appearance of acquired immunity. *J Virol* **77**, 862-870.

Thomssen, R., Bonk, S., Propfe, C., Heermann, K. H., Kochel, H. G. & Uy, A. (1992). Association of hepatitis C virus in human sera with beta-lipoprotein. *Med Microbiol Immunol (Berl)* **181**, 293-300.

Thursz, M., Yallop, R., Goldin, R., Trepo, C. & Thomas, H. C. (1999). Influence of MHC class II genotype on outcome of infection with hepatitis C virus. The HENCORE group. Hepatitis C European Network for Cooperative Research. *Lancet* **354**, 2119-2124.

Timm, J., Lauer, G. M., Kavanagh, D. G., Sheridan, I., Kim, A. Y., Lucas, M., Pillay, T., Ouchi, K., Reyor, L. L., Zur Wiesch, J. S., Gandhi, R. T., Chung, R. T., Bhardwaj, N., Klenerman, P., Walker, B. D. & Allen, T. M. (2004). CD8 epitope escape and reversion in acute HCV infection. *J Exp Med* **200**, 1593-1604.

Todd, S. C., Lipps, S. G., Crisa, L., Salomon, D. R. & Tsoukas, C. D. (1996). CD81 expressed on human thymocytes mediates integrin activation and interleukin 2-dependent proliferation. *J Exp Med* **184**, 2055-2060.

Todres, E., Nardi, J. B. & Robertson, H. M. (2000). The tetraspanin superfamily in insects. *Insect Mol Biol* **9**, 581-590.

Tohami, T., Drucker, L., Radnay, J., Shapira, H. & Lishner, M. (2004). Expression of tetraspanins in peripheral blood leukocytes: a comparison between normal and infectious conditions. *Tissue Antigens* **64**, 235-242.

Tong, M. J., el-Farra, N. S., Reikes, A. R. & Co, R. L. (1995). Clinical outcomes after transfusion-associated hepatitis C. *N Engl J Med* **332**, 1463-1466.

Tortorella, D., Gewurz, B. E., Furman, M. H., Schust, D. J. & Ploegh, H. L. (2000). Viral subversion of the immune system. *Annu Rev Immunol* **18**, 861-926.

Tough, D. F. (2004). Type I interferon as a link between innate and adaptive immunity through dendritic cell stimulation. *Leuk Lymphoma* **45**, 257-264.

Triantafilou, M., Miyake, K., Golenbock, D. T. & Triantafilou, K. (2002). Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. *J Cell Sci* **115**, 2603-2611.

Trinchieri, G. & Valiante, N. (1993). Receptors for the Fc fragment of IgG on natural killer cells. *Nat Immun* **12**, 218-234.

Triyatni, M., Vergalla, J., Davis, A. R., Hadlock, K. G., Foung, S. K. & Liang, T. J. (2002a). Structural features of envelope proteins on hepatitis C virus-like particles as determined by anti-envelope monoclonal antibodies and CD81 binding. *Virology* **298**, 124-132.

Triyatni, M., Saunier, B., Maruvada, P., Davis, A. R., Ulianich, L., Heller, T., Patel, A., Kohn, L. D. & Liang, T. J. (2002b). Interaction of hepatitis C virus-like particles and cells: a model system for studying viral binding and entry. *J Virol* **76**, 9335-9344.

Tsai, S. L., Chen, Y. M., Chen, M. H., Huang, C. Y., Sheen, I. S., Yeh, C. T., Huang, J. H., Kuo, G. C. & Liaw, Y. F. (1998). Hepatitis C virus variants circumventing cytotoxic T lymphocyte activity as a mechanism of chronicity. *Gastroenterology* **115**, 954-965.

Tseng, C. T. & Klimpel, G. R. (2002). Binding of the hepatitis C virus envelope protein E2 to CD81 inhibits natural killer cell functions. *J Exp Med* **195**, 43-49.

Tseng, C. T., Miskovsky, E. & Klimpel, G. R. (2001a). Crosslinking CD81 results in activation of TCRgammadelta T cells. *Cell Immunol* **207**, 19-27.

Tseng, C. T., Miskovsky, E., Houghton, M. & Klimpel, G. R. (2001b). Characterization of liver T-cell receptor gammadelta T cells obtained from individuals chronically infected with hepatitis C virus (HCV): evidence for these T cells playing a role in the liver pathology associated with HCV infections. *Hepatology* **33**, 1312-1320.

Tsitsikov, E. N., Gutierrez-Ramos, J. C. & Geha, R. S. (1997). Impaired CD19 expression and signaling, enhanced antibody response to type II T independent antigen and reduction of B-1 cells in CD81-deficient mice. *Proc Natl Acad Sci USA* **94**, 10844-10849.

Tsuji, K., Feng, M. A. & Wang, D. (2002). Development of human lymphohematopoiesis defined by CD34 and CD81 expression. *Leuk Lymphoma* **43**, 2269-2273.

Tsukiyama-Kohara, K., Iizuka, N., Kohara, M. & Nomoto, A. (1992). Internal ribosome entry site within hepatitis C virus RNA. *J Virol* **66**, 1476-1483.

Turville, S. G., Arthos, J., Donald, K. M., Lynch, G., Naif, H., Clark, G., Hart, D. & Cunningham, A. L. (2001). HIV gp120 receptors on human dendritic cells. *Blood* **98**, 2482-2488.

Ulsenheimer, A., Gerlach, J. T., Gruener, N. H., Jung, M. C., Schirren, C. A., Schraut, W., Zachoval, R., Pape, G. R. & Diepolder, H. M. (2003). Detection of functionally altered hepatitis C virus-specific CD4 T cells in acute and chronic hepatitis C. *Hepatology* **37**, 1189-1198.

Underhill, D. M. (2003). Toll-like receptors: networking for success. *Eur J Immunol* **33**, 1767-1775.

Unutmaz, D. (2003). NKT cells and HIV infection. *Microbes Infect* **5**, 1041-1047.

Urbani, S., Boni, C., Missale, G., Elia, G., Cavallo, C., Massari, M., Raimondo, G. & Ferrari, C. (2002). Virus-specific CD8+ lymphocytes share the same effector-

memory phenotype but exhibit functional differences in acute hepatitis B and C. *J Virol* **76**, 12423-12434.

van Die, I., van Vliet, S. J., Nyame, A. K., Cummings, R. D., Bank, C. M., Appelmelk, B., Geijtenbeek, T. B. & van Kooyk, Y. (2003). The dendritic cell-specific C-type lectin DC-SIGN is a receptor for Schistosoma mansoni egg antigens and recognizes the glycan antigen Lewis x. *Glycobiology* **13**, 471-478. Epub 2003 Feb 2020.

Van Dommelen, S. L. & Degli-Esposti, M. A. (2004). NKT cells and viral immunity. *Immunol Cell Biol* **82**, 332-341.

van Doorn, L. J., Capriles, I., Maertens, G., DeLeys, R., Murray, K., Kos, T., Schellekens, H. & Quint, W. (1995). Sequence evolution of the hypervariable region in the putative envelope region E2/NS1 of hepatitis C virus is correlated with specific humoral immune responses. *J Virol* **69**, 773-778.

van Meer, G. & Sprong, H. (2004). Membrane lipids and vesicular traffic. *Curr Opin Cell Biol* **16**, 373-378.

Van Thiel, D. H., Zhang, X., Baddour, N., Wright, H. I., Friedlander, L. & Gavaler, J. S. (1994). Intrahepatic mononuclear cell populations and MHC antigen expression in patients with chronic hepatitis C [correction of B]: effect of interferon-alpha. *Dig Dis Sci* **39**, 970-976.

Vishnyakova, T. G., Bocharov, A. V., Baranova, I. N., Chen, Z., Remaley, A. T., Csako, G., Eggerman, T. L. & Patterson, A. P. (2003). Binding and internalization of lipopolysaccharide by Cla-1, a human orthologue of rodent scavenger receptor B1. *J Biol Chem* **278**, 22771-22780. Epub 22003 Mar 22721.

Vogt, A. B., Spindeldreher, S. & Kropshofer, H. (2002). Clustering of MHC-peptide complexes prior to their engagement in the immunological synapse: lipid raft and tetraspan microdomains. *Immunol Rev* **189**, 136-151.

Voisset, C., Callens, N., Blanchard, E., Op De Beeck, A., Dubuisson, J. & Vu-Dac, N. (2005). High density lipoproteins facilitate hepatitis C virus entry through the scavenger receptor class B type I. *J Biol Chem* **4**, 4.

Vremec, D., Pooley, J., Hochrein, H., Wu, L. & Shortman, K. (2000). CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J Immunol* **164**, 2978-2986.

Vujanovic, N. L., Nagashima, S., Herberman, R. B. & Whiteside, T. L. (1996). Nonsecretory apoptotic killing by human NK cells. *J Immunol* **157**, 1117-1126.

Wack, A., Soldaini, E., Tseng, C. T. K., Nuti, S., Kimpel, G. R. & Abrignani, S. (2001). Binding of the hepatitis C virus envelope protein E2 to CB81 provides a co-stimulatory signal for human T cells. *European Journal of Immunology* **31**, 166-175.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H. G., Mizokami, M., Bartenschlager, R. & Liang,

T. J. (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* **12**, 12.

Wang, H., Nishanian, P. & Fahey, J. L. (1995). Characterization of immune suppression by a synthetic HIV gp41 peptide. *Cell Immunol* **161**, 236-243.

Wang, Q. M., Hockman, M. A., Staschke, K., Johnson, R. B., Case, K. A., Lu, J., Parsons, S., Zhang, F., Rathnachalam, R., Kirkegaard, K. & Colacino, J. M. (2002). Oligomerization and cooperative RNA synthesis activity of hepatitis C virus RNA-dependent RNA polymerase. *J Virol* **76**, 3865-3872.

Watanabe, S., Kagamu, H., Yoshizawa, H., Fujita, N., Tanaka, H., Tanaka, J. & Gejyo, F. (2003). The duration of signaling through CD40 directs biological ability of dendritic cells to induce antitumor immunity. *J Immunol* **171**, 5828-5836.

Wedemeyer, H., He, X. S., Nascimbeni, M., Davis, A. R., Greenberg, H. B., Hoofnagle, J. H., Liang, T. J., Alter, H. & Rehermann, B. (2002). Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J Immunol* **169**, 3447-3458.

Wei, X., Decker, J. M., Wang, S., Hui, H., Kappes, J. C., Wu, X., Salazar-Gonzalez, J. F., Salazar, M. G., Kilby, J. M., Saag, M. S., Komarova, N. L., Nowak, M. A., Hahn, B. H., Kwong, P. D. & Shaw, G. M. (2003). Antibody neutralization and escape by HIV-1. *Nature* **422**, 307-312.

Weiner, A., Erickson, A. L., Kansopon, J., Crawford, K., Muchmore, E., Hughes, A. L., Houghton, M. & Walker, C. M. (1995). Persistent hepatitis C virus infection in a chimpanzee is associated with emergence of a cytotoxic T lymphocyte escape variant. *Proc Natl Acad Sci USA* **92**, 2755-2759.

Wellnitz, S., Klumpp, B., Barth, H., Ito, S., Depla, E., Dubuisson, J., Blum, H. E. & Baumert, T. F. (2002). Binding of hepatitis C virus-like particles derived from infectious clone H77C to defined human cell lines. *J Virol* **76**, 1181-1193.

Whitney, M., Stack, J. H., Darke, P. L., Zheng, W., Terzo, J., Inglese, J., Strulovici, B., Kuo, L. C. & Pollock, B. A. (2002). A collaborative screening program for the discovery of inhibitors of HCV NS2/3 cis-cleaving protease activity. *J Biomol Screen* **7**, 149-154.

Witherden, D. A., Boismenu, R. & Havran, W. L. (2000). CD81 and CD28 costimulate T cells through distinct pathways. *J Immunol* **165**, 1902-1909.

Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J. & Mathison, J. C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* **249**, 1431-1433.

Wu, J., Song, Y., Bakker, A. B., Bauer, S., Spies, T., Lanier, L. L. & Phillips, J. H. (1999). An activating immunoreceptor complex formed by NKG2D and DAP10. *Science* **285**, 730-732.

Wunschmann, S., Medh, J. D., Klinzmann, D., Schmidt, W. N. & Stapleton, J. T. (2000). Characterization of hepatitis C virus (HCV) and HCV E2 interactions with CD81 and the low-density lipoprotein receptor. *Journal of Virology* **74**, 10055-10062.

Wyatt, C. A., Andrus, L., Brotman, B., Huang, F., Lee, D. H. & Prince, A. M. (1998). Immunity in chimpanzees chronically infected with hepatitis C virus: role of minor quasispecies in reinfection. *J Virol* **72**, 1725-1730.

Xie, Z. C., Riezu-Boj, J. I., Lasarte, J. J., Guillen, J., Su, J. H., Civeira, M. P. & Prieto, J. (1998). Transmission of hepatitis C virus infection to tree shrews. *Virology* **244**, 513-520.

Xu, L. Z., Larzul, D., Delaporte, E., Brechot, C. & Kremsdorf, D. (1994). Hepatitis C virus genotype 4 is highly prevalent in central Africa (Gabon). *J Gen Virol* **75**, 2393-2398.

Xu, Z., Choi, J., Yen, T. S., Lu, W., Strohecker, A., Govindarajan, S., Chien, D., Selby, M. J. & Ou, J. (2001). Synthesis of a novel hepatitis C virus protein by ribosomal frameshift. *Embo J* **20**, 3840-3848.

Yagnik, A. T., Lahm, A., Meola, A., Roccasecca, R. M., Ercole, B. B., Nicosia, A. & Tramontano, A. (2000). A model for the hepatitis C virus envelope glycoprotein E2. *Proteins-Structure Function and Genetics* **40**, 355-366.

Yanagi, M., Purcell, R. H., Emerson, S. U. & Bukh, J. (1997). Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proc Natl Acad Sci USA* **94**, 8738-8743.

Yanagi, M., Purcell, R. H., Emerson, S. U. & Bukh, J. (1999). Hepatitis C virus: an infectious molecular clone of a second major genotype (2a) and lack of viability of intertypic 1a and 2a chimeras. *Virology* **262**, 250-263.

Yanagi, M., St Claire, M., Shapiro, M., Emerson, S. U., Purcell, R. H. & Bukh, J. (1998). Transcripts of a chimeric cDNA clone of hepatitis C virus genotype 1b are infectious in vivo. *Virology* **244**, 161-172.

Yanez-Mo, M., Alfranca, A., Cabanas, C., Marazuela, M., Tejedor, R., Ursa, M. A., Ashman, L. K., de Landazuri, M. O. & Sanchez-Madrid, F. (1998). Regulation of endothelial cell motility by complexes of tetraspan molecules CD81/TAPA-1 and CD151/PETA-3 with alpha3 beta1 integrin localized at endothelial lateral junctions. *J Cell Biol* **141**, 791-804.

Yao, Z. Q., Nguyen, D. T., Hiotellis, A. I. & Hahn, Y. S. (2001). Hepatitis C virus core protein inhibits human T lymphocyte responses by a complement-dependent regulatory pathway. *J Immunol* **167**, 5264-5272.

Yao, Z. Q., Eisen-Vandervelde, A., Waggoner, S. N., Cale, E. M. & Hahn, Y. S. (2004). Direct binding of hepatitis C virus core to gC1qR on CD4+ and CD8+ T cells leads to impaired activation of Lck and Akt. *J Virol* **78**, 6409-6419.

Yarovinsky, F., Zhang, D., Andersen, J. F., Bannenberg, G. L., Serhan, C. N., Hayden, M. S., Hieny, S., Sutterwala, F. S., Flavell, R. A., Ghosh, S. & Sher, A. (2005). TLR11 Activation of Dendritic Cells by a Protozoan Profilin-Like Protein. *Science* **28**, 28.

Yashiro, Y., Tai, X. G., Toyo-oka, K., Park, C. S., Abe, R., Hamaoka, T., Kobayashi, M., Neben, S. & Fujiwara, H. (1998). A fundamental difference in the capacity to induce proliferation of naive T cells between CD28 and other co-stimulatory molecules. *Eur J Immunol* **28**, 926-935.

Yeo, A. E., Ghany, M., Conry-Cantilena, C., Melpolder, J. C., Kleiner, D. E., Shih, J. W., Hoofnagle, J. H. & Alter, H. J. (2001). Stability of HCV-RNA level and its lack of correlation with disease severity in asymptomatic chronic hepatitis C virus carriers. *J Viral Hepat* **8**, 256-263.

Yokoyama, W. M. & Scalzo, A. A. (2002). Natural killer cell activation receptors in innate immunity to infection. *Microbes Infect* **4**, 1513-1521.

Zajac, A. J., Blattman, J. N., Murali-Krishna, K., Sourdive, D. J., Suresh, M., Altman, J. D. & Ahmed, R. (1998). Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med* **188**, 2205-2213.

Zeuzem, S., Lee, J. H. & Roth, W. K. (1997). Mutations in the nonstructural 5A gene of European hepatitis C virus isolates and response to interferon alfa. *Hepatology* **25**, 740-744.

Zeuzem, S., Franke, A., Lee, J. H., Herrmann, G., Ruster, B. & Roth, W. K. (1996). Phylogenetic analysis of hepatitis C virus isolates and their correlation to viremia, liver function tests, and histology. *Hepatology* **24**, 1003-1009.

Zeuzem, S., Diago, M., Gane, E., Reddy, K. R., Pockros, P., Prati, D., Shiffman, M., Farci, P., Gitlin, N., O'Brien, C. B., Lamour, F. & Lardelli, P. (2004). Peginterferon alfa-2a (40 kilodaltons) and ribavirin in patients with chronic hepatitis C and normal aminotransferase levels. *Gastroenterology* **127**, 1724-1732.

Zhang, J., Randall, G., Higginbottom, A., Monk, P., Rice, C. M. & McKeating, J. A. (2004). CD81 is required for hepatitis C virus glycoprotein-mediated viral infection. *J Virol* **78**, 1448-1455.

Zhang, X. A., Bontrager, A. L. & Hemler, M. E. (2001). Transmembrane-4 superfamily proteins associate with activated protein kinase C (PKC) and link PKC to specific beta(1) integrins. *J Biol Chem* **276**, 25005-25013. Epub 22001 Apr 25026.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T. & Chisari, F. V. (2005). Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci U S A* **102**, 9294-9299. Epub 2005 Jun 9296.

Zhu, N., Ware, C. F. & Lai, M. M. (2001). Hepatitis C virus core protein enhances FADD-mediated apoptosis and suppresses TRADD signaling of tumor necrosis factor receptor. *Virology* **283**, 178-187.

Zibert, A., Schreier, E. & Roggendorf, M. (1995). Antibodies in human sera specific to hypervariable region 1 of hepatitis C virus can block viral attachment. *Virology* **208**, 653-661.

Zibert, A., Meisel, H., Kraas, W., Schulz, A., Jung, G. & Roggendorf, M. (1997). Early antibody response against hypervariable region 1 is associated with acute self-limiting infections of hepatitis C virus. *Hepatology* **25**, 1245-1249.

Zignego, A. L., Macchia, D., Monti, M., Thiers, V., Mazzetti, M., Foschi, M., Maggi, E., Romagnani, S., Gentilini, P. & Brechot, C. (1992). Infection of peripheral mononuclear blood cells by hepatitis C virus. *J Hepatol* **15**, 382-386.

Zignego, A. L., Ferri, C., Monti, M., LaCivita, L., Giannini, C., Careccia, G., Giannelli, F., Pasero, G., Bombardieri, S. & Gentilini, P. (1995). Hepatitis C virus as a lymphotropic agent: evidence and pathogenetic implications. *Clin Exp Rheumatol* **13**, S33-37.

Zignego, A. L., Ferri, C., Giannini, C., Monti, M., La Civita, L., Careccia, G., Longombardo, G., Lombardini, F., Bombardieri, S. & Gentilini, P. (1996). Hepatitis C virus genotype analysis in patients with type II mixed cryoglobulinemia. *Ann Intern Med* **124**, 31-34.

Zuckerman, E., Kessel, A., Slobodin, G., Sabo, E., Yeshurun, D. & Toubi, E. (2003). Antiviral treatment down-regulates peripheral B-cell CD81 expression and CD5 expansion in chronic hepatitis C virus infection. *J Virol* **77**, 10432-10436.

Zuckerman, E., Slobodin, G., Kessel, A., Sabo, E., Yeshurun, D., Halas, K. & Toubi, E. (2002). Peripheral B-cell CD5 expansion and CD81 overexpression and their association with disease severity and autoimmune markers in chronic hepatitis C virus infection. *Clin Exp Immunol* **128**, 353-358.